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From the Zoophysiological Laboratory A, and the Institute of Biological Chemistry A,
August Krogh Institute, Copenhagen, Denmark

Properties of a conductive cellular chloride pathway in the skin of the toad (*Bufo bufo*)

By

Erik Hviid Larsen and Poul Kristensen

Received 20 November 1976

Abstract

Hviid Larsen, E. and P. Kristensen. *Properties of a conductive cellular chloride pathway in the skin of the toad* (*Bufo bufo*). Acta physiol. scand. 1978. *102*. 1–21.

Two types of chloride current response to step-wise hyperpolarization of the toad skin is demonstrated. (1) An "instantaneous" response observed immediately upon voltage change, and (2) subsequent slow response, the time course of which is sigmoidal. The slow response is due to an increase of a transcellular conductance which specific to chloride ions. The time constant of the conductance increase is dependent on the amplitude of the transepithelial voltage displacement, the smallest time constants are obtained for the highest amplitudes and are in the order of 30 s. The voltage dependencies of the steady-state conductance and the steady-state chloride current reveal that the chloride pathway has maximum conductance for V 80 mV (outside of the skin being negative) and approaches non-conducting state for V > 0 mV. This strong outward going rectification is steady-state phenomenon. In skins hyperpolarized for few minutes, the "instantaneous" I–V curves show that the chloride pathway in the conducting state allows large inward chloride current (outward chloride flux) to pass in the voltage range 40 mV > V > 0 mV. Calculations based on a three-compartment model indicate that the strong steady-state chloride current rectification cannot be obtained if only the intracellular chloride concentration and the membrane potentials are allowed to vary ("Goldman-rectification"). It is suggested, therefore, that the permeability of the chloride pathway varies reversibly with the transepithelial potential difference. The variable which controls the chloride permeability may be membrane potential or the concentration of an intracellular ion.

In previous paper (Bruus, Kristensen and Hviid Larsen 1976) it was shown that the chloride transport across the skin of the toad *Bufo bufo* is predominantly transcellular. In the electrically short circuited preparation, two different cellular chloride pathways could be distinguished. An exchange diffusion and rheogenic active transport directed towards the inside. We could furthermore show that at clamping voltages larger than or equal to the spontaneous transepithelial electrical potential difference, an acetazolamide (Diamox) sensitive, charge transferring inward transport of chloride exists. Due to this transcellular conductive chloride pathway the spontaneous potential difference across this preparation is only about 20 mV (outside being negative), provided both sides are bathed with NaCl-Ringer's solution.

Analysis of the voltage dependence of unidirectional chloride fluxes revealed that the

steady state chloride net flux (chloride current) is not a linear function of clamping voltage. The present paper deals with a closer examination of this voltage dependent behaviour. By means of transepithelial voltage clamps, the time course of chloride current activation is described and the steady-state voltage conductance relation obtained. The time dependent behaviour and the non-linear steady-state properties of the chloride pathway may originate from changes in intracellular ion concentrations and from changes in membrane potentials of the transporting cells. In order to estimate the significance of such changes for the steady-state current voltage relation, a three-compartment model based on the two-membrane hypothesis is developed for quantitative analysis.

The subsequent paper deals with the relation between the exchange pathway and the conductive pathway for chloride transport in toad skin.

The results presented below have been reported briefly (Hviid Larsen and Kristensen 1976)

Methods

Animals and composition of Ringer solutions. Isolated abdominal skins of the toad *Bufo bufo* (L.) in the middle of the intermoult phase were used throughout this study. Handling of animals in the laboratory prior to experimentation has been described previously (Bruus *et al.* 1976). In the present experiments the skins were always bathed with *NaCl-Ringer's* on the inside, the composition of which is (mmole/liter): Na^+ = 114.0, K^+ = 4, Ca^{++} = 1.0, Cl^- = 114.0, gluconate = 2.0, HCO_3^- = 2.4, and pH = 8.2. The outside medium varied and will be referred to as *NaCl-Ringer*, *Na-gluconate-Ringer* (chloride substituted by gluconate), KCl *Ringer* (sodium substituted by potassium) and *K-gluconate-Ringer* (sodium and chloride substituted by potassium and gluconate, respectively). The experiments were performed at room temperature (20–22 °C).

Experimental set up. For isotope flux measurements, conical lucite chambers with an exposed area of 7 cm² were used (Koefoed Johnsen, Ussing and Zerahn 1952). However many of the expts. presented below were performed by means of a lucite chamber device, similar to the one used by Whittembury (1964). The outer chamber forms an open cup, whose bottom is a 2 cm² exposed skin area resting on a nylon net and tied with a silk thread onto the lucite cylinder which forms the inner chamber. The volume of the solution in the outer chamber is ... ml, and the outside of the skin can be perfused continuously with Ringer's at a flow rate of 2 ml s⁻¹. By manually operating an eight-way stopcock, the perfusion solution can be selected from 8 different flasks. The performance of this set-up was tested by replacing the skin by an anion-selective membrane (Series 100 Amfion). The half time of the potential change after increasing the outside chloride concentration from 10 to 100 mM was found to be ... 1 s. (In this calculation, the logarithmic relation between transmembrane potential and concentration was taken into account, *cf.* Dainty and House 1966.) This response is not a very fast one and it indicates that a considerable, unstirred layer affects the potential response of the anion selective membrane. However with ... toad skin mounted, the response of the short circuit current to sudden removal of outside sodium has considerably longer half times which vary from 5 to 18 s (see Fig. 4 B). Applying the very same procedure ... d with ... frog skin (*Rana temporaria*) mounted in the chamber leads to half time values for short circuit current decrease of 1.7 s. This indicates that ... the toad skin preparation, ... barrier outside the sodium selective membrane prevents very fast exposure of this membrane to the outer bulk solution. This assumption agrees well with the finding that amiloride added to the outer solution blocks the active sodium transport rather slowly ... with ... half time of the decrease in short circuit current of about one minute. This value is 30 times higher than that observed with the frog skin preparation mounted in the same chamber. In view of this finding we did not attempt to improve upon the perfusion conditions.

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3 types of voltage clamp studies were performed. In the first, the skin was allowed to reach the steady-state open circuit potential, the circuit was then switched to and maintained in the voltage clamp mode for a period of time sufficient to obtain steady clamping current. I the second type, the procedure was identical with the first except for superpositioning of square shaped voltage signal of either 20 mV or 50 mV and of 500 ms duration supplied intermittently from the circuit. From the current responses to these voltage displacements, the time course of the skin conductance following voltage clamping was calculated. In the third type of voltage clamp study the skin was clamped at a preselected holding potential. When steady clamping current was obtained, a staircase generator was triggered manually which fed a staircase shaped voltage signal to the command voltage input of the clamp circuit. 10–15 steps of 20 or 10 mV each and of 50 ms duration were used.

The time course of clamping current following voltage clamping was so slow that conventional pen-recorder (RE 511 or RE 520 Servogor) was sufficient to monitor the current response. A higher time resolution was needed to record the current responses to the voltage staircase. For this purpose, transient recorder (DL 901 datalab) and storage oscilloscope (R 5031 Tektronix) were used.

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Amiloride as amiloride HCl was supplied from Merck, Sharp & Dohme.

Note on the leak conductance. At a rather late stage of this study it was realized that the leak conductance in preparations mounted in the perfusion chamber was significantly higher than the previously reported very low paracellular shunt conductance. This difference may be conveniently illustrated by comparing the total skin conductance in preparations mounted in the two types of chambers and exposed to K-gluconate-Ringer outside. In the flux chamber, the conductance values ranged from 0.05 to 0.09 mmho cm^{-2} whereas preparations mounted in the perfusion chamber gave 0.1–0.5 mmho cm^{-2}. The first mentioned values are close to the previously estimated g_{shunt} 0.074 mmho cm^{-2} calculated from isotope flux measurements (Bruus et al. 1976), and indicates that the skin conductance obtained with K-gluconate Ringer outside gives a reasonably good estimate of the paracellular shunt conductance. (A similar method for quick estimate of the paracellular conductance was used by Ussing and Windhager (1964), using K_2SO_4-Ringer instead of K-gluconate Ringer.) The conclusions derived from the experiments below however are not affected by the presence of a larger leak conductance since in both types of preparations the leak pathway has linear current-voltage characteristics with time-invariant conductances in the range -100 mV $< V <$ 50 mV.

Sign notations. When dealing with epithelia, the most widely used current sign notation is to define an inward going current positive. Consequently the transepithelial potential difference (V) should be defined according to $V = \psi_i - \psi_o$, where ψ_o and ψ_i are the potentials of the outer and inner bathing solutions, respectively. These sign notations are used in the present and in the subsequent paper. It is further noted that "hyperpolarization" is used in the sense of increasing the negativity of the outside potential relative to the inside potential.

The quantitative analysis of the three-compartment model was performed on a Digital Equipment PDP8/e.

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Non-steady-state current response to voltage clamp

The response of transepithelial current to stepwise changes in transepithelial voltage from the resting value to a predetermined value is shown in Fig. 1. The records were obtained as follows. The skin was equilibrated at open circuit conditions until a steady resting potential was achieved. The voltage was then stepped to the desired value, and the time course of the clamping current was recorded. After a 5–10 min period of voltage clamping, the skin was again allowed to relax at open circuit conditions for a period of time sufficient to bring the spontaneous potential difference back to its initial resting value. The procedure was repeated for the 5 subsequent clamping voltages indicated. In the example shown in Fig. 1 the steady-state resting potential between the sequential voltage clamping periods, was -22 to -24 mV. In other words, the non-steady-state phenomena observed upon voltage clamping are reversible.

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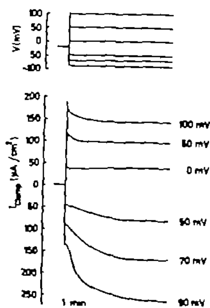


Fig. 1 Records of clamping current (I_{clamp}) following a step change in transepithelial potential difference (V). For each record V was changed from its steady-state spontaneous value to the clamping voltage indicated. Steady-state spontaneous value of V = −22 to −24 mV. The records were obtained from one toad skin preparation exposed to NaCl-Ringer's on both sides.

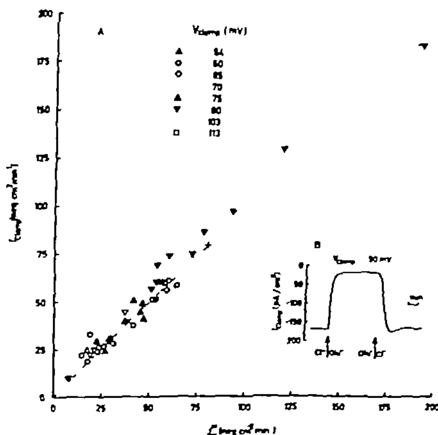


Fig. — A. Plot of steady-state outward clamping current (I_{clamp}) versus simultaneously measured steady-state chloride influx (J_{Cl}^{in}). Each point represents an average of two consecutive half hour periods at the clamping voltage indicated and with NaCl-Ringer's bathing both sides of the skin. Total number of skins 40. The dashed curve indicates the line of identity. B. Response of outward clamping current to substitution of outside chloride by gluconate. Half time of current decrease following removal of outside chl... 6 sec.

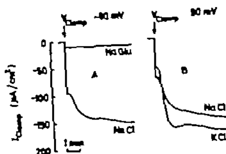


Fig 3 *A* Time course of clamping current following voltage clamp at −90 mV in the presence of chloride and gluconate outside, respectively Note: with gluconate outside, I_{clamp} is time-invariant. *B* Same as in A, but with different cations outside. With Na^+ as well as with K outside, I_{clamp} displays similar time-dependent behaviour

Typically the outward current increases with time upon hyperpolarization and the inward current decreases with time for clamping voltages above zero mV In neither case is the time course of the current response simple. For example, the half time of current change is dependent on voltage. Also note that the outward current shows a marked initial inflexion for large negative voltage values. The subsequent paragraphs of this paper deal with an investigation of the time-variant behaviour of the clamping current recorded during hyperpolarization.

Contribution of Cl^--influx to outward clamping current

The relation between outward clamping current and inward movement of chloride ions was established by chloride isotope flux measurements. Fig. 2 A shows all simultaneous measurements of steady-state outward clamping currents and steady-state chloride influxes calculated from the inward movement of ^{36}Cl It can be seen that at these voltages the influx of chloride closely matches the outward current flux, although the steady-state clamping currents obtained from different skin preparations vary by a factor of more than ten. This equivalence of clamping current and chloride influx in skins bathed on both sides with NaCl-Ringer's implies that at $V < -55$ mV the clamping current is governed by the conductance of a pathway selectively allowing chloride ions to pass. It is expected, therefore, that substitution of chloride by e.g. gluconate in the solution bathing the outside of the skin will significantly reduce the steady-state outward clamping current. Fig. 2 B shows that this is the case

As a simple approach towards an identification of the ionic components involved in the non-steady-state response to voltage clamp, anion and cation substitutions in the outer bath were performed. Fig 3 A shows the time course of clamping current recorded from skins exposed to chloride-Ringer's and to chloride-free-Ringer's outside. When the external chloride concentration is reduced to zero, the instantaneous outward current is reduced by one order of magnitude, and the time dependence of the outward current completely disappears. Substitution of sodium ions by potassium ions in the solution bathing the outside of the skin is expected to reduce cellular sodium activity (Koefoed-Johnsen and Ussing 1958). This treatment, however does not affect the main characteristics of the outward current response to maintained hyperpolarization, cf Fig. 3 B. These results strongly suggest that the time variant behavior of the outward current is restricted to a pathway selectively passing chloride ions.

TABLE I Effect of amiloride on chloride influx and outward clamping current. Skins bathed in NaCl Ringer's on both sides. Amiloride added to the outer solution. All fluxes and current-values are averages of two consecutive half hour periods and are expressed in nmol cm^{-2} min^{-1}

Animal no	Spontaneous potential mV	J_{Cl}^{in}	Clamping potential mV	Control J_{Cl}^{in}	Control I_{clamp}	5 μM amiloride J_{Cl}^{in}	5 μM amiloride I_{clamp}
1	−14	14.9	−60	24.6	−21.3	22.2	[illegible]
2	−13	14.3	−60	59.5	−52.9	58.5	[illegible]
3	−11	12.9	−60	3‿3	−31.1	29.0	[illegible]
4	−14	16.7	−60	62.9	−64 ‿	69.6	[illegible]

Effect of amiloride

Amiloride added to the solution bathing the outside of the amphibian skin inhibits sodium transport across the outer sodium selective membrane (Dörge and Nagel 1970 Cuthbert and Shum 1974 Morel and Leblanc 1975 Fuchs, Hvid Larsen and Lindemann 1977) As can be seen from Table I neither the steady-state chloride influx nor the steady-state clamping current observed at $V_{clamp} = -60$ mV are affected by exposing the skin to 5 μM amiloride outside. The insensitivity of inward chloride flux to amiloride was also demonstrated in another type of experiment the result of which is shown in Fig. 4 A Substitution of chloride by gluconate in the solution bathing the outside of the skin reduces outward clamping current from −188 μA/cm to −20 μA/cm Neither the steady-state clamping current nor the decrease ($-I_{Cl}$) observed after reducing the outer chloride concentration to zero mM is significantly affected by the presence of 60 μM amiloride in the outer solution. This concentration of amiloride, however abolishes the active sodium flux (Fig. 4 B) The short circuit current is almost entirely carried by sodium ions, as demonstrated by its reduction towards

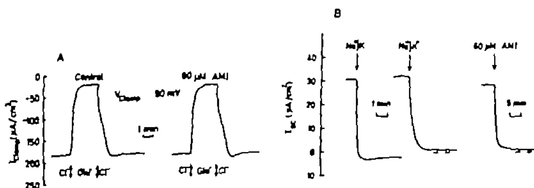


Fig. 4 *A*. Response of outward clamping current (V_{clamp} = − 90 mV) t chloride-gluconate ubstit tion in outside bathing solution. Left and right hand records were obtained from the same kin preparation bathed with N Cl-Ringer s on both sides, the latter however fter 25 min. of exposure t 60 μM am loride outside Neither the mplitude of current decrease (I_{Cl}) nor the half time of current response ($T_{\frac{1}{2}}$) is ffected by the presence of amiloride. $I_{Cl} = -168$ μA/cm² $T_{\frac{1}{2}} = 12$ s.

Fig. 4 *B* Response of short circuit current (I_{sc}) upon Na K ubstitution in o tside bathing solution Two left hand record illustrate the difference of a "fa t and a slow respond g preparation ($T_{\frac{1}{2}} = 5$ and 18 s, respecti ely). R ght hand panel show the response of short circuit current t dd tion of 60 μM miloride outside. Note the d fferent time scale. $T_{\frac{1}{2}}$ 60 s

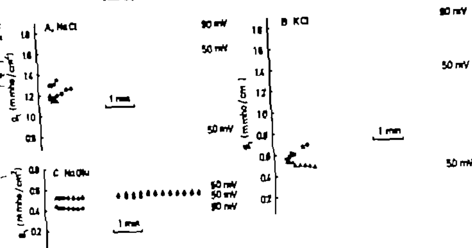


Fig. 5. Time course of total skin conductance (g_t) following step voltage clamp from the steady-state spontaneous potential difference. A. NaCl-Ringer' outside. B. KCl-Ringer' outside. C. Na-gluconate-Ringer' outside.

zero upon substitution of outside sodium by potassium. 60 µM amiloride almost completely inhibits this inward active sodium flux (right hand part of Fig. 4 B).

Voltage-conductance relations

The results presented in the preceding sections have shown that hyperpolarization of the skin brings about a stimulation of an outward current carried by an inward transport of chloride ions. In another series of expts. the time course of total skin conductance and the voltage dependence of the steady-state conductance were investigated. Like in the experiments presented above, the skin was pre-equilibrated under open circuit conditions, when a constant open circuit potential was achieved, the potential was changed in a step-wise fashion and maintained at the new value for several minutes. The clamping current was recorded and the skin conductance measured intermittently by recording the current response to a square voltage pulse of 20 or 50 mV amplitude and of 500 ms duration. These intermittent conductance measurements do not affect the overall current time course.

The time course of conductance change is illustrated by representative examples in Fig. 5. When exposed to NaCl- or KCl-Ringer's but not to Na-gluconate Ringer's outside, the conductance is time variant upon a step change of transepithelial voltage. Especially for large negative values of V_{clamp}, the time course of conductance change is clearly sigmoidal. Upon depolarization to 50 mV the conductance decreases in skins exposed to NaCl-Ringer's outside. This response is not typical with KCl-Ringer's outside, however with potassium outside, the spontaneous potential is about zero mV as compared to −25 mV with sodium outside.

The time it takes for the conductance to reach its new steady value is dependent on the amplitude of voltage change. In order to facilitate comparison of the rate of conductance increase for different step voltage changes, the time required to reach half maximal conductance change, $T_{½}$, will be used. Table II summarizes $T_{½}$ values obtained from skins under

T BLE II $T_½$ values (for def nition af $T_½$ see text) of conductance increase after step voltage change froi spontaneous potential to a preselected clamping potential.

Animal no	Spontaneous potential mV	$T_½$, seconds Clamping potential, mV →	−90	−80	−70	−50	−30
1	−19 to −26		39	42	42	60	102
2	−24 to −27[a]		48	53	55	66	95
	−2[b]		50	54	53	70	90
3	− 4 to −28[a]		40	—	51	108	—
4	−22 to −.3[a]		60	—	95	120	—
5	−17[a]		33	—	50	55	—
5	−2[c]		28	—	60	120	—
6	−16 to −19[a]		60	—	70	105	—
7	−27 to −.8[a]		40	—	80	90	—

NaCl Ringer s outside.
[b] NaCl-Ringer's outside + 60 μM milloride.
KCl Ringer s outside.

different experimental conditions. Although the absolute values for equal voltage conditi vary from skin to skin, the general picture is quite uniform. $T_½$ ranges from about 30 120 s, the smallest values invariably are obtained for the largest command voltage steps

Steady-state conductance values (g_t^∞) are reached between t = 3 min and t = 5 min, depe ing on voltage. In Fig. 6 the variation of steady state conductance with clamping volt under different experimental conditions is depicted In the following these curves are deno g_t^∞–V curves The g_t^∞–V curve for skins exposed to NaCl-Ringer s outside is S-shaped, w a maximum g_t^∞ at V well below the resting potential and the minimum above zero mV (F 6 A and B) The main characteristics of the g_t^∞–V curve is not affected by application 60 μM amiloride outside, or by substitution of outside sodium by potassium (Fig. 6 C and l This gives strong support to the view that the sigmoidal g^∞–V relation is not due to sodi channel processes, but is a characteristic feature of a chloride conducting pathway The v different g^∞–V curve obtained with a chloride free outer solution supports this hypothe (Fig. 6 F).

Ouabain added to the solution bathing the inside of the frog skin reduces the active tra epithelial sodium transport by inhibiting the active Na/K pump located at the inward fac membrane (Koefoed-Johnsen 1957 Dörge and Nagel 1972) Addition of ouabain to to skin likewise inhibits, although slowly the active sodium transport In the skin from whi the results shown in Fig. 6 E are obtained, the short circuit current was reduced from 18 μ cm to 1 μA/cm after 3 h of exposure to 10^{-4} M ouabain However even after this treatme the S-shaped g_t^∞–V curve was retained.

Relation between steady state chloride current and transepithelial potential difference

Steady-state current values are depicted as a function of clamping voltage in Fig. 7 A, B a C. These steady-state I–V curves are non-linear and show outward going rectification can be deduced from the g_t^∞–V relations discussed above. Blocking the sodium channels w amiloride interferes selectively with the transcellular sodium current Another way of elir

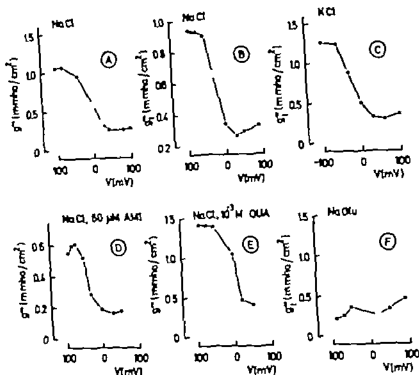


Fig. 6. Examples of steady-state total skin conductance (g^T) as function of transepithelial clamping voltage (V). The outside Ringer' solutions are indicated for each curve. Results from the amiloride treated skin were obtained 30 min after addition of the drug to the outer solution. Ouabain was added to the inner bathing solution and the measurements performed after 3 h of drug exposure.

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$$I_{clamp} = I_{Cl} + V\,g_{shunt} \qquad (1)$$

where I_{clamp} is the steady-state clamping current at voltage V, g_{shunt} is the shunt conductance, and I_{Cl} is the steady-state chloride current through the voltage dependent cellular pathway. For each skin, g_{shunt} may be approximated by the skin conductance obtained with K-gluconate Ringer's outside (*cf.* p. 3). Taking I_{clamp} values from curves B and C of Fig. 7 and using the g_{shunt} estimated as described above, we have calculated the steady-state current voltage curve of the chloride conducting pathway (see Fig. 7 E and F). Another method of determining the I_{Cl}–V relation was suggested in a previous paper (Bruus *et al.* 1976). By means of isotope flux measurements, the steady-state clamping current for different voltages was split into its individual ionic components. The I_{Cl}–V relation obtained by this analysis is reproduced in Fig. 7 D. By these different ways of estimating the I_{Cl}–V curve of the cellular path-

TABLE II $T_{\frac{1}{2}}$ values (for definition af $T_{\frac{1}{2}}$ see text) of conductance increase after step voltage change fr spontaneous potential to a preselected clamping potential

Animal no.	Spontaneous potential mV	$T_{\frac{1}{2}}$ seconds				
		Clamping potential mV ... −90	−80	−70	−50	−30
1	−19 to 26	39	42	42	60	102
2	24 to −27[a]	48	53	55	66	95
2	−?[b]	50	54	53	70	90
3	−24 to −28[a]	40	—	51	108	—
4	−22 to −23[a]	60	—	93	120	—
5	17[a]	33	—	50	55	—
5	−2[c]	28	—	60	120	—
6	16 to −19[a]	60	—	70	105	—
7	−27 to −28[a]	40	—	80	90	—

NaCl-Ringer s outside.
[b] NaCl Ringer s outside + 60 μM amiloride
KCl Ringer s outside.

different experimental conditions. Although the absolute values for equal voltage condr vary from skin to skin the general picture is quite uniform. $T_{\frac{1}{2}}$ ranges from about 30 120 s, the smallest values invariably are obtained for the largest command voltage step.

Steady-state conductance values (g_t^{∞}) are reached between t = 3 min and t = 5 min, depending on voltage. In Fig. 6 the variation of steady-state conductance with clamping voltage under different experimental conditions is depicted In the following these curves are denoted g_t^{∞}–V curves. The g_t^{∞}–V curve for skins exposed to NaCl Ringer s outside is S-shaped, with a maximum g_t^{∞} at V well below the resting potential and the minimum above zero mV (Fig 6 A and B) The main characteristics of the g_t^{∞}–V curve is not affected by application of 60 μM amiloride outside, or by substitution of outside sodium by potassium (Fig. 6 C and D). This gives strong support to the view that the sigmoidal g_t^{∞} V relation is not due to sodium channel processes, but is a characteristic feature of a chloride conducting pathway The very different g^{∞}–V curve obtained with a chloride free outer solution supports this hypothesis (Fig. 6 F)

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Relation between steady state chloride current and transepithelial potential difference

Steady state current values are depicted as a function of clamping voltage in Fig. 7 A B and C. These steady-state I–V curves are non-linear and show outward going rectification as can be deduced from the g_t^{∞}–V relations discussed above. Blocking the sodium channels with amiloride interferes selectively with the transcellular sodium current Another way of elim-

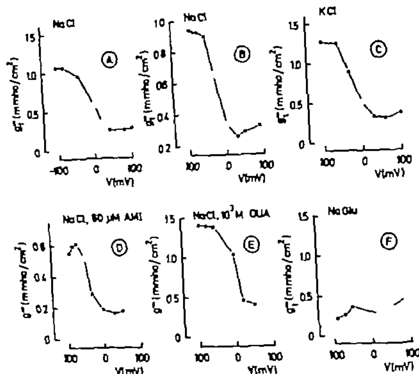


Fig. 6. Examples of steady-state total skin conductance (g_T^{ss}) as function of transepithelial clamping voltage (V). The outside Ringer' solutions are indicated for each curve. Results from the amiloride treated skin were obtained 30 min after addition of the drug to the outer solution. Ouabain was added to the inner bathing solution and the measurements performed after 3 h of drug exposure.

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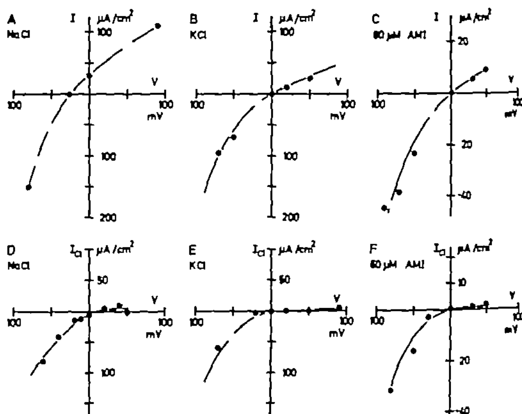


Fig. 7 *A*, *B* and C. Steady-state current voltage relations of toad skin exposed to *A* NaCl Ringer's outside, *B*, KCl-Ringer's outside, and *C*, NaCl Ringer's outside with 60 μM amiloride. *D*, *E* and *F* Steady-state current voltage relation of the cellular chloride conducting pathway. *D* I_{Cl} calculated from isotope flux measurements, data from Bruus *et al* 1976. *E* and *F* I_{Cl} obtained from steady-state total currents corrected for the shunt component (B, E. g_{shunt} = 0.50 mmho cm⁻² C, F g_{shunt} = 0.14 mmho cm⁻²). For further explanation see text.

way we arrive at essentially the same result namely that the cellular conductive chloride pathway exhibits a pronounced outward going rectification (in terms of current) with practically zero chloride current for V ~ 0 mV This means that the steady-state flux of chloride through this pathway becomes negligible as the transepithelial potential difference is decreased and reversed.

"Instantaneous" relation between current and transepithelial potential difference

In a series of experiments, the skin was continuously clamped on a preselected transepithelial potential difference, the holding potential When a constant clamping current was attained the voltage was changed in a staircase fashion, in 10–15 steps of 20 or 10 mV and of 50 ms duration, while the resulting current responses were recorded. Accordingly the current voltage curve obtained by this method is recorded within a time interval 1–2 orders of magnitude shorter than T_1 of the time dependent conductance changes described above. We can therefore assume that the steady-state chloride conductance prevailing at the holding potential is not significantly influenced by the voltage staircase applied Examples of such "instantaneous" I–V curves are shown in Fig. 8 The current now varies linearly with voltage. Note that the "instantaneous" I–V curve obtained from a holding potential of about −

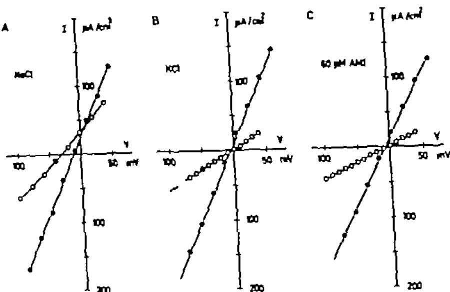


Fig. 8 "Instantaneous" current-voltage relations of toad skin. *A* NaCl-Ringer' outside, ● Holding potential, V_H = −90 mV ○ V_H −40 mV *B* KCl-Ringer' outside, ● V_H = −95 mV ○ V_H = −40 mV *C*. NaCl-Ringer' with 60 μM amiloride outside, ● V_H = −97 mV ○, V_H = −40 mV

is linear even for *inward* currents, and that this relationship prevails in sodium-free outside bathing solution and in skins exposed to 60 μM amiloride outside. Furthermore from Fig. 8 B and C it can be seen that only a minor fraction of this current goes through the shunt pathway as is apparent from the "instantaneous" I V curves displayed from a holding potential of −40 mV in which situation the cellular chloride conductance is close to zero (Fig. 7 D E, and F). These results show therefore, that the activated conductive chloride pathway does not rectify and that the inward current through the activated pathway is carried by an *outward* flux of chloride ions across the membranes facing the outer bulk solution.

Discussion

In this and in an earlier paper (Bruus, Kristensen and Hvid Larsen 1976) we have presented experimental evidence that the potential difference across toad skin affects the chloride conductance of a cellular pathway. Important features of this potential dependence have been recognized in the present study:

Upon a step-wise change of the transepithelial potential difference, the change in chloride conductance towards its new steady state value proceeds slowly with a rise time of the order of minutes. The time dependent behaviour is not simply exponential, but exhibits an initial delay. The rise time of the conductance change depends on the magnitude of the voltage change, it is reduced as the step voltage change is increased. Furthermore, we have shown that the relation between steady-state chloride conductance and transepithelial potential difference is S-shaped. Highest conductance values are observed at potential differences more negative than the spontaneous potential whereas the chloride conductance approaches zero

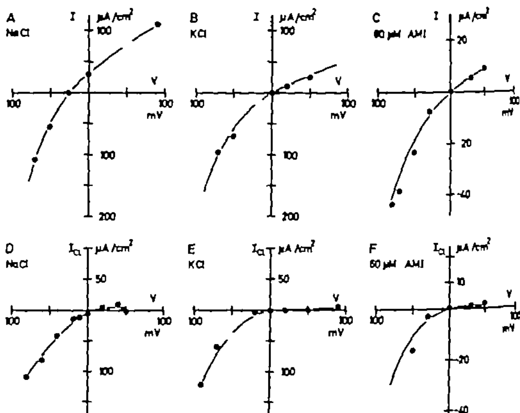


Fig. 7 *A*, *B* and *C* Steady-state current-voltage relations of toad skin exposed to *A*, NaCl-Ringer's outside, *B* KCl-Ringer outside and *C* NaCl-Ringer's outside with 60 μM amiloride. *D*, *E* and *F* Steady-state current voltage relation of the cellular chloride conducting pathway. *D* I_{Cl} calculated from isotope flux measurements, data from Bruus *et al* 1976. *E* and *F* I_{Cl} obtained from steady-state total currents corrected for the shunt component (B, E g_{shunt} = 0.50 mmho cm⁻² C, F g_{shunt} = 0.14 mmho cm⁻²). For further explanation see text.

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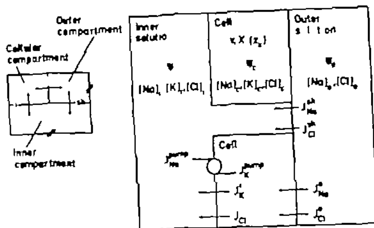


Fig. 9. *Left*: Diagrammatical representation of the three-compartment model. The compartments are separated by the physical barriers denoted, *o*: outward facing membrane, inward facing membrane, and sh: shunt membrane. The inner and outer compartments are assumed infinitely large. *Right*: Outline of the symbolical representation of the model. *v*: cell volume. $X_c(z_x)$: amount of intracellular, non-diffusible molecules with the mean valency z_x. Ψ: electrical potentials. J: fluxes. Concentrations are indicated by brackets.

Here, $[Cl]_o$ and $[Cl]_c$ are the outer and cellular chloride concentrations, V the outer membrane potential ($\psi - \psi_o$), u_{Cl} the molar mobility of chloride within the membrane, β the partition coefficient, and δ the membrane thickness. F, R, and T have their usual significance. A similar equation may be assumed to describe J_{Cl}^{i}. It is now easily recognized that a non-linear steady-state I_{Cl}–V curve may be due to the voltage dependence of $[Cl]_c$ in combination with the fact that the membrane potentials vary. Another, and less trivial possibility would be that P_{Cl} changes in response to changes in membrane potential ("gating"). As an approach to estimating the significance of the "trivial" possibilities, we shall calculate theoretical current–voltage curves of an epithelial cell layer provided with properties of the anuran skin.

A three-compartment model

The quantitative analysis will be based upon the three-compartment model shown in Fig. 9. This representation of the transporting cells is in accordance with the two-membrane hypothesis (Koefoed-Johnsen and Ussing 1958, Ussing and Windhager 1964) supplied with a number of simplifying assumptions:

(a) Fluxes of only three ionic species are dealt with, sodium, potassium and chloride. Since all other ions are present at relatively low concentrations their contributions to the membrane currents are ignored.

(b) Intracellularly a number of larger molecules (X_c) are present which cannot pass the surface membrane of the cell. Their mean valency is z_x.

(c) The K⁺-permeability of the outward facing membrane and the Na⁺-permeability of the inward facing membrane are assumed zero. Electrophysiological studies (Koefoed-Johnsen and Ussing 1958, Lindley and Hoshiko 1964, Fuchs *et al.* 1977) as well as studies of the cell volume response to osmotic variations and Na⁺–K⁺ substitutions of the bathing solutions (MacRobbie and Ussing 1961) show that the K⁺-movement through the outer membrane and the passive Na⁺-movement across the inner membrane is at least one order of magnitude slower than that of other penetrating ions. Furthermore, the transepithelial potassium flux and the passive component of the transepithelial sodium flux seem to follow an extra-cellular route (Mandel and Curran 1972, Brown *et al.* 1976). As a consequence of these findings, we have decided to ignore completely the passive sodium current across the inner membrane and the potassium current across the outer membrane.

(d) All of the transcellular chloride current is assumed to pass through the sodium transporting cells. One type of evidence has been discussed on p. 12 in this paper. Further evidence will be given elsewhere (Kristensen 1978).

The strategy of the analysis is now to find proper equations for the individual membrane currents including the kinetics of the active Na/K-pump, subsequently to specify mathematically the steady-state conditions, and finally to solve the equations for selected values of the independent variables. The equa-

as the potential difference is decreased and reversed. This gives rise to a steady-state I_{Cl}–V characteristics exhibiting a pronounced rectification with almost zero steady-state net flux of chloride in outward direction. The rectifying property however is a steady-state phenomenon. In a situation where the transcellular chloride conductance is maximum, the instantaneous cellular current varies linearly with the transepithelial potential difference, and the I–V curve proceeds with the same slope for currents carried by an inward and outward flux of chloride ions across the outer limiting membranes of the transporting cells.

The cellular chloride conducting pathway is insensitive to amiloride and shows the same voltage dependent behaviour when the sodium channels are blocked by amiloride. Therefore, the cellular chloride conducting pathway operates in parallel to the amiloride blockable sodium channels of the outward facing membrane

By comparing the time courses of chloride and sodium currents to a sudden change in the outside concentration, the position of the cells through which the chloride current passes can be determined relative to the sodium transporting cells. The chloride current response to removal of outside chloride is exemplified in Figs. 2 B and 4 A, whereas the sodium current response to reduction of outside sodium concentration to zero mM is shown in Fig. 4 B. It can be seen that the decrease in chloride current is as fast as the decrease in sodium current, although the half times of these current responses vary from skin to skin. The reason for these rather slow and varying current responses is obscure. It is unlikely that they are due to the presence of a simple unstirred layer on top of the skin (see methods) Without overrating the resolution of this type of experiment, the results show however that the chloride pathway is located to cells within a distance from the outer bulk solution comparable to that of the sodium transporting cells. In other words they cannot be close to the subepidermal gland cells which are assumed to contribute to the adrenaline stimulated (outward) chloride transport (Koefoed-Johnsen *et al* 1952)

The variables which may affect the transcellular chloride conductance

In the experimental approach presented here, the ionic composition of the solutions bathing the inner and the outer surface of the skin—as well as the transepithelial potential difference—are fully controlled by us. Cellular compartments within the skin, however may change ionic composition and volume during voltage clamping. Therefore, in considering the nature of the voltage dependence of the transcellular chloride conductance, we have to take into account that variables other than the transepithelial potential difference may change when the skins are submitted to voltage clamping.

A transcellular chloride current is governed by two conductances in series, i.e. the conductance of the outward facing membrane (g^o_{Cl}), and that of the inward facing membrane (g^i_{Cl}). Thus, the transcellular chloride conductance (g_{Cl}) is given by

$$g_{Cl} = g^o_{Cl} \cdot g^i_{Cl}/(g^o_{Cl} + g^i_{Cl}) \tag{2}$$

The individual membrane conductances are *functions of ion concentrations of the bathing solutions,* membrane potential, and the mobility of the ion within the membrane. Even for the most simple case—the homogenous membrane with a linear electrical potential profile—this dependence comes out as a rather complex equation (see f.ex. Finkelstein and Mauro 1963). Taking g^o_{Cl} as an example and considering the sign convention used in the present paper this dependence reads

$$g^o_{Cl} = \frac{P^o_{Cl} F^2 V}{R\ T} \frac{[Cl]_o - [Cl]_c\,\xi_o}{(1 - \xi_o)\ln([Cl]_o/[Cl]_c\,\xi_o)} \tag{3}$$

where $\xi_o = \exp(-FV_o/(RT))$, and $P^o_{Cl} = \omega_{Cl} RT\beta/\delta$

TABLE III. Computed dependent variables of interest for the discussion for 3 values of the transepithelial clamping voltage. The independent variables used in these computations are listed in the legend to Fig. 10. The open circuit potential, $V_{open} = -31.5$ mV

V (mV)	−100	0	+50
I_{clamp} ($\mu A\ cm^{-2}$)	−124.1	35.2	77.4
I_{Cl} ($\mu A\ cm^{-2}$)	−125.6	0	22.8
g_{Cl}^{o} (mmho cm^{-2})	2.00	1.00	0.92
V_o (mV)	2.8	78.4	115.3
$[Cl]_c$ (mM)	15.9	5.4	5.6
J_i (nmol cm^{-2} min^{-1})	1.6	9.0	17.9

changes is expected to increase the chloride conductance of the outward facing membrane. Our calculations show that these changes taken together result in an increase of the transcellular chloride conductance by a factor of 2.8, despite that P_{Cl}^{o} and P_{Cl}^{i} are kept constant. By itself this is a noteworthy result, because it illustrates that a strong asymmetry may be inherent in a cellular chloride pathway even if this pathway is assumed to be of the most simple nature, and the skin is exposed to Ringer's of identical composition on its two sides.

From Fig. 10 B, however, it can be seen that the S-shaped g_{Cl}^{o}–V relation typically observed (Fig. 6) cannot be reproduced by the model. Another significant deviation from the observed data is that the very large negative chloride currents for V < −70 mV presuppose such large chloride permeabilities that also the chloride current for V > 0 mV amounts to a relatively large fraction of the total current. In the example shown in Fig. 10 A, $I_{Cl} = 23\ \mu A/cm^2$ at V = 50 mV. In contrast, our measurements show that the steady-state chloride current in a toad skin clamped to this voltage is not significantly different from zero. In other words, the observed strong outward going rectification of the cellular chloride pathway cannot be obtained from this simple model. It is likely, therefore, that a chloride permeability varies reversibly with the transepithelial potential difference, i.e. it increases at hyperpolarizing clamping voltages and decreases following depolarization and reversal of the transepithelial potential difference. This possibility will be considered further in the following section.

Evidence of a chloride permeability which varies reversibly with the transepithelial potential difference

In the computed example discussed above, the unidirectional chloride fluxes come out to be about 9 nmol cm^{-2} min^{-1} under short circuit conditions. Chloride fluxes of this magnitude are within the range of fluxes observed in the toad skin clamped to zero mV. These observed chloride fluxes, however, do not seem to be of a simple passive nature as is assumed in the computations. The first point of interest here is that both the chloride influx and outflux is practically uninfluenced by the transepithelial potential difference in the range 0 mV < V < 50 mV (Bruus et al., 1976, Fig. 6). This observation together with the dependence of the chloride outflux on the chloride concentration of the outer solution led us to suggest that the larger fraction of the chloride outflux passes the skin by way of a Cl–Cl exchange. In Table IV is collected steady-state fluxes measured in skins from 10 different toads clamped at V = 50 mV

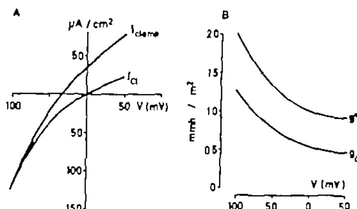


Fig. 10 *A* Computed voltage dependence of the steady-state transepithelial current (I_{clamp}) and the steady-state transcellular chloride current (I_{Cl}) *B* Computed g^{∞}–V and g_{Cl}^{∞}–V relations. For these calculations the following independent variables were used. $[Na]_o = [Na]_i = 115$ mM $[Cl]_o = [Cl]_i = 117$ mM $[K]_i = 2$ mM. Permeability coefficients and pump constants (10^{-6} cm s^{-1}) P^{o} = 1 $P^{o}_{Cl} = 12$, $P^{i}_{K} = 50$, $P^{i}_{Cl} = 25$, P^{o}_{K} 0 $P^{i}_{Cl} = 0.08$ $A_{N} = 9$ $A_{K} = 6$. $X_o = 30$ nanomole $z_x = -1$ eqv mole $\sigma_{KCl} = 0.95$ T = 295 K.

tions and the computer analyses are treated in the *Appendix* to this paper where also the validity of the assumptions underlying the various equations is discussed. Here we shall proceed with the representation and discussion of the results of the analysis.

Theoretical steady-state I–V curves obtained under the assumption of constant permeabilities

During the initial examination of the transport model we tried several combinations of permeability coefficients and analysed for the shape and position of the steady-state I–V curve. It soon became clear that only for combinations of permeability coefficients where $P_{Cl} > P$ and $P^{i}_{Cl} < P^{i}_{K}$ the I–V curve is non linear with a large I_{Cl} component at hyperpolarizing voltages. With this restriction, the absolute values of the permeability coefficients were selected in order to obtain approximately (1) the experimentally determined short circuit current, (2) the measured open circuit potential, and (3) a ratio of R /R 0 8, where R and R are the outer membrane and the transcellular resistance, respectively This last mentioned assumption agrees with results of micro-electrode studies of frog skin exposed to NaCl Ringer's on both sides (Ussing and Windhager 1964 Lindemann and Thoms 1967).

Examples of computed I–V curves are shown in Fig 10 The legend to Fig. 10 contains the independent variables on which these calculations are based. The dependent variables of interest for our discussion are given for three selected clamping voltages in Table III

Comparison of Fig. 10 with Fig. 7 reveals that the model reproduces I–V curves displaying outward going rectification with a large I_{Cl} component at hyperpolarizing clamping voltages which resemble those obtained in the *in vitro* preparation. From the values given in Table III it can be seen that in the voltage range 50 mV to −100 mV the chloride conductance increase is due to (1) a decrease and a reversal of the outer membrane potential, and (2) a relatively large increase in $[Cl]_c$. By comparing with eqn. (3) it is seen that each of these

House (1971) investigated the response of the transepithelial potential difference toad skin to changes in ion concentrations of the outer solution, and he concluded that the chloride permeability of the outside border is larger than its sodium permeability However the assumption that $P^{i} < P^{i}_{K}$ may not be realistic In the frog skin epithelium the K distribution across the inner membranes is far from thermodynamic equilibrium (MacRobbie & Ussing 1961). Calculations show that this requires P^{i} P^{i}

during the non-steady-state period following a step voltage change. By itself this increase in $[Cl]_c$ is expected to increase the conductance of the cellular chloride pathway (*cf.* equ. (3)). It cannot be assumed, therefore, that the conductance changes shown in Fig. 5 are caused by permeability changes only. This complication also makes it difficult to identify the variable which controls the chloride permeability. The permeability activation may be initiated by a change in membrane potential, or by a change in the concentration of an intracellular ion in which case the ion must be assumed to participate in a permeability controlling reaction step. From the type of experiments presented in this paper we cannot distinguish between these possibilities.

Appendix

The variables of the model presented in Fig. 9 of the discussion section are divided into independent and dependent variables, see the listing in Table I A and Table II A. The interrelationship between these variables will be explained below.

Equations of passive fluxes

Throughout we will assume that the passive fluxes of Na+, K+ and Cl− are given by the Nernst-Planck equation integrated under the assumption of a constant electrical field within the individual surface membranes (Goldman 1943, Hodgkin and Katz 1949). With the voltage and current sign notation used in the present paper the integrated flux equations read:

(1) *Passive fluxes through the paracellular shunt pathway*

$$J_j^{sh} = \frac{P_j^{sh}(z_j FV/(RT))}{1-\exp(z_j FV/(RT))}([j]_i - [j]_o \exp(z_j FV/(RT))), \tag{1 A}$$

here $V = \psi_i - \psi_o$ and j is substituted by either Na+ or Cl−.

(2) *Passive fluxes through the outward facing membrane*

$$J^o = \frac{P_j^o(z_j FV_o/(RT))}{1-\exp(z_j FV_o/(RT))}([j]_c - [j]_o \exp(z_j FV/(RT))), \tag{2 A}$$

here $V_o = \psi_c - \psi_o$ and j is either Na+ or Cl−.

(3) *Passive fluxes through the inward facing membrane*

$$J_j^i = \frac{P_j^i(z_j F(V-V_o)/(RT))}{1-\exp(z_j F(V-V_o)/(RT))}([j]_i - [j]_c \exp(z_j F(V-V_o)/(RT))), \tag{3 A}$$

Here j = either K+ or Cl−.

For small variations of V the paracellular fluxes in frog skin and toad skin are governed by eqn. (1 A) (Mandel and Curran 1972, Bruus *et al.* 1976). For large positive values of V (reversal of the transepithelial potential difference) P_{Na}^{sh} and P_{Cl}^{sh} are not constant. In the voltage range of interest for the present analysis, however, eqn. (1 A) is a fairly good approximation. Also, it has been experimentally verified that the Na+ transport across the outer membrane in frog skin takes place according to eqn. (2 A). The use of this equation, however, requires that $[Na]_o$ is kept constant, which is fulfilled here. If $[Na]_o$ varies P_{Na}^o must be replaced by $P_{Na,max}^o \cdot K_m/(K_m + [Na]_o)$, where $P_{Na,max}^o$ = the Na+-permeability corresponding to $[Na]_o = 0$ mM and K_m = an empirical constant (Fuchs *et al.* 1977). A dependence of P_{Na}^o on $[Na]_c$ (Baker, 1971; Hviid Larsen 1973; Erlij and Smith, 1973; Morel and Leblanc, 1975) is not taken into account here. The application of the formalism above to J_K^i, J_{Cl}^i and J^o has not yet been experimentally investigated. The use of eqns. (2 A) and (3 A), however, is not invalidated by our hypothesis that the larger fraction of the transcellular Cl− flux goes via an exchange mechanism. For the calculation of current-voltage curves, intracellular ion concentrations, and cell volume, a 1:1 chloride exchange [illegible] not appear in the equations, since these are supposed "net-fluxes.

TABLE IV Steady-state chloride influx (J^{in}_{Cl}) and outflux (J^{out}_{Cl}) through the isolated toad skin exposed to NaCl-Ringer's on both sides and clamped at V = 50 mV In each experiment influx and outflux were obtained from laterally symmetrical skin halves from one toad. Data taken from Bruus et al 1976.

Animal no	J^{in}_{Cl} nmol cm^{-2} min	J^{out}_{Cl}	J^{in}_{Cl}/J^{out}_{Cl}	I_{Cl} μA cm^{-2}
1	4.0[illegible]	3.60	1 1	−0.68
2	4 60	5.57	0 83	1.56
3	4.[illegible]1	8.44	0.50	6 80
4	5.57	4 93	1 13	−1.03
5	2.57	3.28	0.78	1 14
6	2.73	2.96	0 9	0.37
7	4 44	5.27	0.84	1.33
8	3.93	3.56	0.73	−0 60
9	3.59	5.38	0 69	[illegible].88
10	3.35	3 63	0 92	0.45
Mean			0 85	1.2[illegible]
±S E.			0.06	0.73

If the chloride fluxes are of a simple passive nature the flux ratio is given by (Ussing, 1949)

$$\frac{J^{in}_{Cl}}{J^{out}_{Cl}} = \frac{(Cl)_o}{(Cl)_i} \exp\left(\frac{-FV}{RT}\right)$$

where $(Cl)_o$ and $(Cl)_i$ are the chloride activities in the outer and inner bathing solutions Under the prevailing experimental conditions the flux ratio is predicted to be 0.14 The experimentally determined flux ratio, however amounts to 0 85 ± 0 06, which is significantly different from 0 14 (P < 0 05 %) On the contrary the experimentally determined flux ratio is only slightly smaller than one, which is expected for a cellular Cl Cl exchange in parallel to a low conductance extracellular route. Thus, the result of this flux ratio analysis is consistent with the hypothesis that an exchange mechanism governs the transcellular chloride transport for $V_{clamp} > 0$ mV In other words, the steady-state permeability of the conductive cellular pathway must be close to zero in this voltage range

As revealed by the "instantaneous" I–V curves shown in Fig. 8 the conductive chloride pathway allows a large inward chloride current (outward chloride flux) to pass if the skin has been hyperpolarized for a few minutes. We therefore conclude that the steady-state permeability of the conductive chloride pathway is a function of the transepithelial potential difference.

The time course of conductance change

The kinetics of the permeability activation are probably reflected in the time course of total conductance increase following hyperpolarization of the skin (Fig. 5) Thus, the activation reaction seems to be a slow process, proceeding with a time constant of no less than 20 s. This makes it very likely that the concentration of chloride in the cellular compartment increases

Note that the voltage sign notation in the paper quoted is opposite to the one used in the presen

Steady-state criteria

These concern the requirements of constant (time-independent) concentration of any of the intracellular components, electroneutrality and osmotic equilibrium between the intracellular and the inner compartment. Mathematically these criteria are expressed by the following equations.

(1) *Constant intracellular concentrations*

$$\Sigma J_j^o - \Sigma J_j^i = 0, \qquad (6\ A)$$

where the summation is taken over the individual pathways, i.e. the total flux of the ion j across the outer membrane is equal to the total flux of this ion across the inner membrane.

(2) *Electroneutrality*

$$[Na]_c + [K]_c + z_x X_c/v - [Cl]_c = 0 \qquad (7\ A)$$

(3) *Osmotic equilibrium*

The epithelial cell layer of frog skin swells if the tonicity of the solution bathing the inner border is lowered. In contrast, the epithelial cell volume (in SO_4 Ringer's) is independent on the tonicity of the outer solution (MacRobbie and Ussing 1961). Accordingly we shall assume that the cells at steady-state are osmotically equilibrated with the inner solution. The fact that both P_K^i and P_{Cl}^i is different from zero implies that KCl probably exhibits a reflection coefficient (σ_{KCl}) which is different from unity. Taking this into account, the requirement of osmotic equilibrium between the cellular and the inner compartment leads to the following equation

$$[K]_c + [Na]_c + (2\sigma_{KCl} - 1)[Cl]_c + X_c/v = [Na]_i + (2\sigma_{KCl} - 1)[K]_i + [Cl]_i \qquad (8\ A)$$

Combination of eqns. (7 A) and (8 A) gives the expression of the steady-state volume of the transporting cell

$$v = \frac{(1 - z_x) X_c}{2(\sigma_{KCl} - 1)[K]_i + 2[Cl]_i - \sigma_{KCl}[Cl]_c} \qquad (9\ A)$$

The inner membrane reflection coefficient for KCl has not been experimentally determined. In the example presented in the discussion section we have arbitrarily assumed $\sigma_{KCl} = 0.95$. Choosing $\sigma_{KCl} = 0.5$ however has only a slight effect on the dependent variables of interest here. Thus, the conclusions drawn in the discussion section are not influenced by our choice of σ_{KCl}.

Procedure of numerical solution

Proper combination of the above equations leads to an equation of the form,

$$F(V_o) = 0, \qquad (10\ A)$$

which is transcendental in V and, therefore, cannot be solved analytically. For its numerical solution we used Newton's method of successive approximations, where the difference between a new guess ($V^{(n+1)}$) to eqn. (10 A) and the immediately preceding one ($V^{(n)}$) is given by:

$$V^{(n)} - V^{(n)} - (F(V_o)/F'(V_o))_{V = V^{(n)}},$$

where $F'(V_o)$ is the first derivative of $F(V_o)$. The iteration was stopped when:

$$|V^{(n)} - V^{(n)}| < 2.5 \cdot 10^{-4}\ mV$$

A closer examination of the function $F(V_o)$ revealed that it has only one solution for each set of independent variables.

After the correct value of V is found the other dependent variables listed in Table II A are calculated for that V. It is easily recognized, however that a large number of other steady-state parameters can be calculated too. The steady-state clamping current (I_{clamp}) and the steady-state cellular chloride current are given by:

$$I_{clamp} = z_{Na} F(J_{Na}^o + J_{Na}^{sh}) - z_{Cl} F(J_{Cl}^o - J_{Cl}^{sh}) \qquad (11\ A)$$

$$I_{Cl} = z_{Cl} F J_{Cl}^o \qquad (12\ A)$$

which of course are of particular interest for the present study

TABLE I A. Listing of the independent variables of the model.

Symbol	Definition
$[Na]_o$, $[Cl]_o$	Concentration of Na and Cl^- in outer compartment
$[Na]_i$, $[Cl]_i$, $[K]_i$	Concentration of Na Cl and K in inner compartment
P^{sh}_{Na}, P^{sh}_{Cl}	Na and Cl^- permeability coefficient of shunt membrane
P^o_{Na}, P^o_{Cl}	Na^+ and Cl^- permeability coefficient of outer membrane
P^i_K, P^i_{Cl}	K and Cl permeability coefficient of inner membrane
A_{Na}, A_K	*Pump coefficients defined by eqns. (4 A) and (5 A)*
X_c	Amount of intracellula non-diffusible molecules
z_x	Their mean valency
σ_{KCl}	KCl reflection coefficient of inner membrane
V	Transepithelial potential difference
T	Temperature in K

Throughout the standard symbols for the Faraday (F), gas constant (R) and valency of the ion species j (z_j) are used

Active fl es through the in ard facing membrane

The general form of the pump flux equations will be

$$J^{pump}_{Na} = A_{Na}\,[Na]_c \tag{4 A}$$

$$J^{pump}_{K} = -A_K[Na]_c \tag{5 A}$$

where A_{Na} and A_K are positive constants. As is seen, the active flux of N is assumed proportional to the intracellula N -concentration This is a good appro imation in the oltage range V < 0 mV here $[Na]_c$ is small. I cases where P^o_{Na} is high nd V > 0 mV the pump saturates. Saturation, however is not expected to occu w thin the range of $[N\,]_c$ variation of nterest for the present nalysis. (A model analysis based upon saturation kinetics of the Na/K pump is discussed n Hvid Larsen 1978.) F rthermore, we assume a fixed coupl ng ratio of 3 2 between the active Na -flux and the ctive K -fl (Thomas 1972). E idence for varying Na K coupling ratio has been btained by Brinley and Mullins (1974) working with the squid axon. Using other ratios of p mp fluxes taken from their study however does not affect the conclusions emphasized in the discussion Finally we assume that the active transport mechanism operates independently of the inner membrane potential This assumption is valid fo the squid axon for ariation of V between −10 and −90 mV (Brinley and Mullins 1974). It should be added that due to the high resistance of the outer membrane as compared t that of the inner one, the variation of the nner membrane potential is expected to be small and about one order of magnitude less than the   ltage cha ges mposed on the skin

TABLE II A Listi g of the dependent variables used in this paper

Symbol	Definition
$[Na]_c$, $[Cl]_c$, $[K]_c$	Concentration of Na Cl and K in the cellular compartment
J^{sh}_j, J^o_j, J^i_j	Passive fluxes of j through the shunt (*sh*), outer ( ), nd inner ( ) membrane
I^{sh}_j, I^o_j, I^i_j	*Membrane currents of j through the membranes sh o, a d*
I_{clamp}	Total transepithelial current
g^{sh}_j, g^o_j, g^i_j	Integral conductance of the ionic pathw ys of the membranes *sh* nd *i*
g_{to}	Total transepithelial conductance
g_c	Transcellular chloride conductance
E^{sh}_j, E^o_j, E^i_j	Equ librium potential of j across the membranes *sh o* nd *i*
J^{pump}_{Na}	Active flu of Na through the inner membrane
J^{pump}_K	Active flux of K through the inner membrane
V	Outer membrane potential ($-\psi$ ψ_o)
	Cell water olume
J^{ept}_{Cl}	Transepithelial unid rectional outflux of Cl^-
J^{in}_{Cl}	Transepithelial unidirectional i flux of Cl^-

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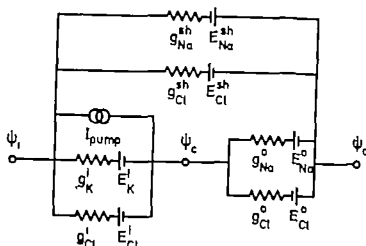


Fig. A 1 Electrical circuit analogue of the three-compartment model. The upper indices to the conductances and electromotive forces indicate, o outward facing membrane, i inward facing membrane, and sh shunt membrane. The rheogenic Na/K pump located in the inward facing membrane is equivalized to a current source. The pump current (I_{pump}) is dependent on the cellular Na -concentration, but independent of the inner membrane potential.

The electrical circuit analogue and conductance equations

The choice of electrical circuit representing the three-compartment model depends on the structure of the membranes (Finkelstein and Mauro 1963) W will assume that each of the limiting membranes is mosaic, which means that it consists of regions exclusively permeable to Cl and other regions exclusively permeable to Na. It follows from this assumption that each of the ionic current components can be ascribed its own driving force and conductance. Accordingly the mixed equivalent circuit show in Fig. A 1 will be used. It follows from the depicted geometrical arrangement of conductors, that *the steady-state transepithelial conductance* (g_t^∞) is given by

$$g_t^\infty = g^{sh}_{Na} + g^{sh}_{Cl} + \frac{(g^o_{Na} + g^o_{Cl})(g^i_K + g^i_{Cl})}{g^o_{Na} + g^o_{Cl} + g^i_K + g^i_{Cl}} \tag{13 A}$$

For each choice of independent variables and subsequent determination of dependent variables, the value of the individual ion conductances (the integral conductance, g_j) is calculated from

$$g_j = I_j/(V_m - E_j),\ V_m \neq E_j, \tag{14 A}$$

where $I_j (= z_j F J_j)$ is the membrane current carried by j, E_j the equilibrium potential of j calculated from the Nernst-equation, and V_m the membrane potential.

For the sake of completeness, we shall recall that for an ionic pathway with constant field properties, and provided $E_j \neq 0$, the instantaneous I_j–V curve is non-linear which means that the integral conductance, g is voltage dependent For the case of g^o_{Cl} this dependence is expressed mathematically in eqn (3) on p 13 Note that eqn. (14 A) is not defined for $V_m = E_j$. With the constant field assumption and continuing with g^o_{Cl} as the example, it can be shown that

$$g^o_{Cl} = \frac{P^o_{Cl} F^2 V_c [Cl]_o [Cl]_o}{R\ T^2([Cl]_o - [Cl]_c)}\quad V \neq E^o_{Cl},\ [Cl]_c \neq [Cl]_c \tag{15 A}$$

Our thanks are due to Mr Ole Bengtson for constructing and to Mr Benny Andström for servicing the voltage clamp circuits, and to Dr Bjørn Rasmussen for his help during the development of the computer program. The fast voltage clamp circuit provided with staircase function was constructed and built for us by Abteilung für Membranforschung an Epithelien, Physiologie (II) der Universität des Saarlandes, Homburg/Saar BRD It is pleasure to thank professor Bernd Lindemann for allowing us to benefit from the expertise of his laboratory Technical assistance by Mrs Lone Pedersen and Mrs Lisbet Sorikjær is appreciated. Purchase of electronic equipment was supported by a grant from the Danish Natural Science Research Council.

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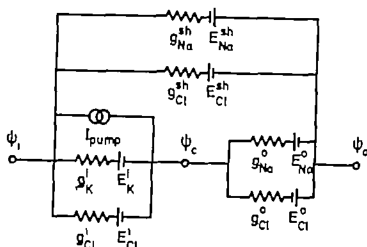


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$$g_{Cl}^{o} = \frac{P_{Cl}^{o} F^2 V_o [Cl]_c [Cl]_o}{R\ T\ ([Cl]_c - [Cl]_o)}\ \ V = E_{Cl}^{o},\ [Cl]_c \neq [Cl]_o \tag{15 A}$$

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It was the purpose of the experiments described below to obtain additional information on the possibility of a common molecular basis for the chloride exchange diffusion pathway and the pathway responsible for the conductive transfer of chloride under hyperpolarization. This was achieved by comparing the effects of some inhibitors and of ionic substitutions on chloride efflux under short circuit conditions with the effects of these inhibitors and ionic substitutions on clamping current during hyperpolarization.

Some of the experiments have already been reported recently (Kristensen and Hvid Larsen 1976).

Methods

The experimental details, including storage and treatment of the toads (*Bufo bufo*) and the chloride flux measurements were described in a previous communication (Bruus *et al.* 1976)

The chloride Ringer solution had the following composition. 111.5 mM Na^+, 1.9 mM K^+, 0.9 mM Ca^{++}, 114.8 mM Cl^- and 2.4 mM HCO_3^-. Bromide, iodide, nitrate, and gluconate Ringer solutions were prepared with salts of these anions instead of chloride.

Phloretin was obtained from K & K Laboratories (USA), and furosemide was kindly supplied by Hoechst Danmark A/S

Transepithelial potential differences are given in this paper as $V = \psi - \psi$

Results

In the experiments to be described we compare 2 modes of chloride transport across the isolated toad skin. A chloride-chloride exchange diffusion and a chloride transport with charge transfer. The exchange diffusion is studied under short circuit conditions, and can be measured quantitatively as the decrease in ^{36}Cl efflux observed, when the outside solution is changed from chloride to sulphate Ringer's. The conducting pathway is studied under conditions, where the transepithelial potential difference is clamped at values more negative than the spontaneous potential difference, which is about −20 mV in toad skins exposed to sodium chloride Ringer's outside. The cellular chloride current can then be estimated in two ways, either by measuring the influx of ^{36}Cl, or simply by recording the outward clamping current, since it was shown in the preceding paper (Hvid Larsen and Kristensen 1977) that for $V_{clamp} < -35$ mV the hyperpolarizing current is almost entirely carried by an inward transport of chloride.

The relations between the two types of chloride transport are studied by comparing their sensitivities to inhibitors and to substitution of chloride by other anions in the outer bathing medium.

Neither anion substitutions nor inhibitors can be assumed *a priori* to affect specifically the chloride pathways. In the present experiments, therefore, short circuit current was recorded in order to disclose effects on active sodium transport.

The efflux of chloride into an outside bathing solution of NaCl-Ringer's varies considerably from skin to skin, ranging in the present material from 1 to about 40 nmol cm⁻² min⁻¹. This efflux consists of two components, a cellular exchange diffusion component and a paracellular shunt component (Bruus *et al.* 1976). The shunt flux, however, is small and of constant magnitude, ranging from 0.6 to 1.0 nmol cm⁻² min⁻¹ in the short circuited sk… large reduction of chloride efflux caused by various treatments as presented below m…

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Relation between chloride exchange diffusion and a conductive chloride pathway across the isolated skin of the toad (*Bufo bufo*)

By

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Abstract

Kristensen P and E. Hviid Larsen *Relation between chloride exchange diffusion and a conductive chloride pathway across the isolated skin of the toad* (*Bufo bufo*) Acta physiol. scand 1978 *102* 22–34

Substitution of chloride in the outside bathing medium of the toad skin with bromide, iodide, nitrate and sulphate leads to a reduction in the apparent exchange diffusion of chloride across this tissue, and also to a reduction of the chloride current recorded during hyperpolarization. A series of inhibitors (thiocyanate, furosemide, phloretin, and acetazolamide) also affects chloride exchange diffusion, hyperpolarization current as well as chloride influx during hyperpolarization. Although in some cases, effects on short circuit current were also observed, none of the effects on chloride transport systems could be explained as secondary effects due to a primary interaction with the sodium transport mechanisms. A correlation was found between the clamping current recorded during hyperpolarization and the efflux of chloride under short circuit conditions with chloride Ringer on both sides. On the basis of these findings, and the results reported in the previous paper (Hviid Larsen and Kristensen 1977) it is considered probable that the membrane molecules responsible for chloride exchange diffusion under short circuit conditions, are rearranged under the influence of a hyperpolarizing clamping voltage, thereby forming channels allowing charge transferring transport of chloride.

The main fraction of transepithelial chloride transport across toad skin occurs *via* cellular pathways (Bruus *et al* 1976) In a previous paper the conductive pathway was characterized with respect to its voltage and time dependence. In skins clamped at a transepithelial voltage difference exceeding the spontaneous potential difference, the electrical properties of the epithelium were found to be governed by a cellular chloride conducting pathway (Hviid Larsen and Kristensen 1977) Under short circuit conditions, however the main fraction of chloride transport seems to occur via an electrically silent chloride exchange diffusion (Bruus *et al* 1976)

Since it was found (Bruus *et al* 1976) that acetazolamide partially inhibits transcellular conductance as well as chloride exchange diffusion, it is possible that common molecular structures are involved in both mechanisms of chloride transport

TABLE II. The effect on clamping current of anion substitutions in the outside bathing solution. Inside solution is chloride Ringer's, inward current is considered positive.

Exp no	Clamping voltage mV	Clamping current ($\mu A\ cm^{-2}$)		
		Cl^- Ringer's	Br^- Ringer	I^- Ringer's
1	80	− 44 3	− 21 9	+ 2.5
	80	75 0	− 50 0	+ 2 9
3	80	94 3	− 78 6	+ 4.3
4	110	61 4	− 55.7	− 2.1
5	−110	18 6	− 15.7	−11 4
6	110	− 57 1	− 39.3	−10 7
7	−110	371 4	−207.1	−32 9
8	−110	4 3	− 19.3	− 5.7

(Bruus *et al.* 1976), and the effluxes are equal to those measured with sulphate or gluconate Ringer's on the outside, *i.e.* they are estimates of the extracellular electro-diffusional transport of chloride, which was characterized in a previous communication (Bruus *et al.* 1976). Also substitution with bromide Ringer's leads to a significant reduction in chloride efflux, but in all skins, the remaining chloride efflux is larger with bromide on the outside than with nitrate. From this observation we conclude that bromide can exchange with chloride across the toad skin, but that it is not as good an exchange partner as chloride itself. The effluxes measured with iodide Ringer on the outside cannot be distinguished from those measured with nitrate Ringer's, indicating that iodide does not exchange significantly with chloride. With respect to their ability to exchange with chloride, the anions tested can therefore be listed in the following order: Cl > Br > I ≃ NO

Under short circuit conditions, anion substitutions not only affect chloride efflux, but moreover the short circuit current (I_{sc}) which is significantly increased, see Fig. 1. With respect to their effect on I_{sc} the anions rank in the reverse order of their exchange capability. This observation was made in all 8 expts. of Table I. The spontaneous transepithelial potential difference measured with different anions in the outside solutions was: Chloride Ringer's, 24.3 mV bromide Ringer's, −39.4 mV iodide Ringer's −72.0 mV and nitrate Ringer's, 77.0 mV (averages of measurements in expts. of Table I).

The clamping currents measured during hyperpolarization in the above expts. are given in Table II. Clearly the largest currents are observed, when the outside is bathed with chloride Ringer's. The currents measured with iodide are very much smaller. Bromide holds an intermediate position, giving about 2/3 of the current observed with chloride in the outside bathing medium. Fig. 2 shows that amiloride, added to the outer solution, does not alter the clamping currents obtained with different anions in the outer solution. This shows that a cellular sodium current does not contribute significantly to the outward clamping currents. The ranking order of the halides with respect to carrying current through the skin, therefore, is the same as that obtained when comparing their exchange capabilities.

The positive clamping currents observed in few cases in Table II occur when the spontaneous potential difference in iodide Ringer ... more negative than the preselected clamping voltage

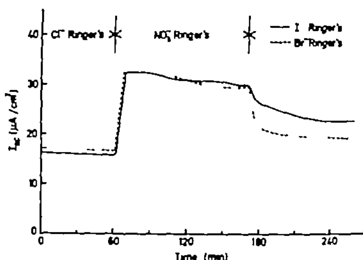


Fig. 1 Response of short circuit current to changes of the anion of the outside bathing medium. Tw laterally symmetrical half skins were used. The outside anion was changed from chloride, to nitrate, to bromide in the ne half skin, and from chloride, t nitrate, to iodide i the other half skin. The simul taneously measured chloride effluxes of this and seven similar experiments are given in Table I

therefore be interpreted as inhibition of the chloride exchange component Any effect of various drugs on the shunt flux is easily detected by measuring the outflux towards chloride free outer solution in the presence of the drug. Such control flux measurements with sulphate Ringer s outside were performed in nearly all experiments, and the results will be given when they are considered essential.

External anion substitution

In the first series of expts. the chloride efflux was measured in short circuited skins exposed in consecutive periods to (1) Cl Ringer s, (2) NO Ringer s, and (3) Br⁻ Ringer s or I Ringer s, see Fig. 1 After completion of the flux measurements of each of the three periods the skins were hyperpolarized for 5 min which is sufficient to attain the steady-state clamping current.

The results concerning the chloride effluxes under short circuit conditions are presented in Table I With nitrate Ringer s on the outside, exchange diffusion of chloride is absent

TABLE I The effect on chloride efflux of changing the nion of the outside bathi g solution The skins are short circuited, and the inside is bathed w th hloride Ri ger s.

Exp. no	Chloride efflux (neq cm⁻² mi)			
	Cl⁻ Ringer s	Br⁻ Ringer s	I⁻ Ringer	NO Ringer's
1	6.2	2.5	0 89	1.00
2	4.8	2.6	0 74	0.70
3	11 4	3.8	0.54	0.55
4	5 6	3 0	0.57	0 62
5	7.9	1.3	1 10	0 75
6	4 0	1 7	0 55	0.88
7	34 7	5 6	1 70	1.60
8	1 7	1 4	1.20	0 91

TABLE IV *The effect of thiocyanate on chloride influx and simultaneously recorded clamping current in skins clamped at −80 mV. The skins used are the laterally symmetrical halves of those used for efflux measurements given in Table III. Concentration of SCN⁻ is 10 mM on both sides.*

Exp. no	Chloride influx neq. cm⁻² min⁻¹		Clamping current given as equivalent ionic flux (neq. cm⁻² min⁻¹)	
	Control	With SCN⁻	Control	With SCN⁻
1	21.4	10.7	33.3	9.8
2	37.3	26.5	44.4	20.5
3	60.5	36.7	49.3	42.2
4	7.7	4.8	10.2	2.1
5	16.6	9.3	22.2	7.5

The effects of thiocyanate on chloride influx and simultaneously recorded clamping current during hyperpolarization are presented in Table IV. It appears that both parameters are reduced considerably by the addition of SCN⁻.

The effect of thiocyanate on the short circuit current recorded during chloride efflux measurements, is exemplified in Fig. 3 showing that the short circuit current is increased in the presence of 10 mM SCN⁻ in both bathing solutions.

Furosemide

Furosemide has been shown to inhibit chloride transport in the ascending limb of Henle's loop in the rabbit kidney (Burg *et al.* 1973). Table V shows the effect of this drug on chloride movements across toad skin. It can be seen that chloride efflux is reduced significantly, the remaining steady state efflux being only a little larger than the efflux usually measured with sulphate Ringer's on the outside.

In the hyperpolarized skin, furosemide reduces chloride influx and outward clamping current to about 50% of the respective values observed in the control period (Table V).

Fig. 4 summarizes the effect of furosemide on the short circuit current in the six experiments presented above. A small but transient increase of this parameter is observed.

Phloretin

The effect of phloretin added simultaneously to inside and outside bathing solutions is presented in Table VI. In four out of the 5 expts., a significant chloride exchange diffusion



Fig. 3. Three examples of short circuit current recordings showing the effect of SCN⁻ (10 mM, both sides).

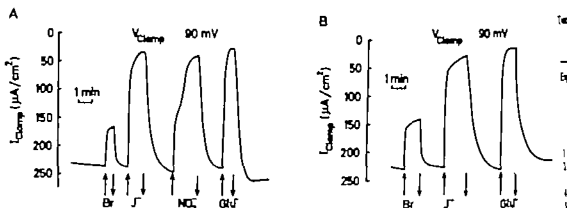


Fig. 2. An experiment showing the changes in clamping current obtained by sequential changes of the anion in the outside bathing medium, in the absence (A), and in the presence (B) of 60 μM amiloride.

Inhibition by thiocyanate

Thiocyanate was shown to inhibit chloride transport in the frog skin (Kristensen 1972). Therefore the effect of this ion on chloride movements and clamping current in the toad skin was investigated. The effect of 10 mM SCN on the apparent exchange diffusion under short circuit conditions and on chloride influx under hyperpolarization was studied on skins from the same animal, one half skin being used for efflux measurements, the other for measurements of influx and clamping current Table III shows the effect of SCN on chloride efflux. In some skins, the efflux measured in the absence of the inhibitor is already rather small which is often the case during the winter months. But by comparing these chloride effluxes with those obtained with sulphate Ringer s on the outside, it is possible to obtain a measure of the apparent exchange flux of chloride which is equal to the decrease in chloride efflux caused by changing the anion in the outside bathing medium from chloride to sulphate. Thus, by comparing columns 4 and 6 of Table III it can be seen that SCN inhibits chloride exchange diffusion across the toad skin.

TABLE III The effect of thiocyanate on chloride efflux nder short circuit conditi ns. The apparent chloride exchange diffusion is obtained by subtracting the efflux measured with sulphate Ringer s on the outside from that measured with chloride Ringer s on the outside. SCN was added as NaSCN to a final concentration of 10 mM on both sides.

Exp no.	Chloride fluxes (neq cm^{-2} min)				
	Efflux measured with chloride outside	Efflux measured with sulphate outside	Apparent chloride exchange diffusion without SCN	Efflux measured with SCN added	Apparent chloride exchange diffusion with SCN$^-$ dded
1	1.2	0.66	0 54	0.74	0.08
2	4.0	0.70	3.30	2.50	1.80
3	11 1	1.00	10.1	5 00	4 00
4	1.2	0.53	0.67	0.52	0 0
5	0 9	0 53	0.37	0.51	0 0

VI The effect of phloretin (0.25 mM on both sides) on chloride efflux under short circuit conditions, on clamping current during hyperpolarization, and on spontaneous transepithelial potential difference. Short circuit current values were obtained from the automatic recordings of the current during chloride efflux measurements. Measurement of chloride efflux into sulphate Ringer's gives estimates of the free diffusion shunt.

sp	Chloride efflux neq cm⁻² min⁻¹			Clamping current µA cm⁻² (clamping voltage −110 mV)		Spontaneous PD mV		Short circuit current µA cm⁻²	
	Sulphate Ringer's outside	Chloride Ringer's outside Control	Chloride Ringer' outside Phloretin	Control	Phloretin	Control	Phloretin	Control	Phloretin
	0.94	5.5	3.1	−91.4	60.0	−17	−39	10.8	9.1
	0.86	2.8	1.6	−57.1	−31.4	−21	−38	6.4	6.4
	0.65	3.5	1.4	40.7	−25.0	−21	−34	6.4	6.5
	1.71	2.0	2.3	−17.9	20.0	−30	−32	9.0	7.1
	2.16	8.8	3.7	−171.4	−21.4	−14	−25	12.1	11.3

In Fig. 5 hyperpolarization current is plotted against the chloride efflux measured under short circuit conditions. The results are divided into 2 graphs. On the right for clamping voltages between 100 mV and −110 mV and on the left for clamping voltages from 70 mV to 80 mV. In both cases a highly significant correlation is observed, although the scatter appears to be more pronounced at *higher clamping voltages*.

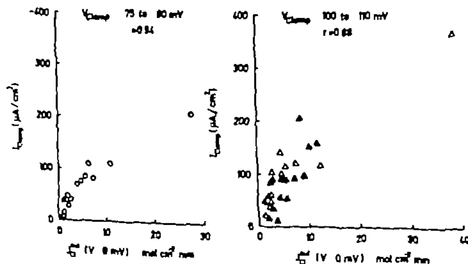


Fig. 5 The relation between chloride exchange diffusion and clamping current. Each point represents an experiment in which chloride efflux and hyperpolarization current were measured on the same skin preparation. The abscissa gives the total efflux not corrected for the contribution made by the extracellular shunt pathway which normally amounts to less than 1 neq. cm⁻² min⁻¹. A correlation analysis showed that the variables, I_{Clamp} and J_{Cl}^{out} are correlated, correlation coefficients, r = 0.94 and r = 0.88, respectively (hypothesis ρ = 0 gives P < 0.0005 for each group).

TABLE V The effect of furosemide (2 mM on both sides) on chloride efflux under short circuit condi and on clamping current and chloride influx under hyperpolarization (clamping oltage, 80 i Fluxes and currents are given in neq cm^{-2} min^{-}

Exp no.	Chloride efflux under short circuiting		Chloride influx under hyperpolarization		Clamping current recorded during hyperpolarization	
	Control	Furosemide	Control	Furosemide	Control	Furosemide
1	27 7	2.4	118.0	69 1	131	68
2	6.4	1 4	54 1	22.6	69	16
3	1.8	1 1	44 1	13.0	56	12
4	13.5	1 4	72.1	33 7	76	30
5	15 8	3.9	77.8	36 5	87	33
6	5.3	0.9	53 4	25.5	61	23

is evident. In these expts., phloretin inhibits the efflux of chloride under short circuit co tions as well as the clamping current under hyperpolarization In one experiment (no Table VI) these inhibitions are absent In this skin the exchange component is rather s (compare chloride efflux measured with chloride and sulphate outside, respectively) the measured clamping current can easily be accounted for by a comparatively larger diffusion shunt pathway of this skin preparation.

The short circuit current is not affected by the presence of phloretin, it remained a control value for at least one hour after the addition of the drug.

In the 4 expts. in which chloride movements are affected by phloretin, an increase in spontaneous transepithelial potential difference is observed upon addition of the drug.

Correlation between exchange diffusion flux and outward clamping current density

It is evident from the experimental results that the chloride exchange diffusion path and the conductive chloride pathway have properties in common. An interrelation between these two pathways may also be disclosed by a correlation analysis relating chloride effluxes ($\simeq$ exchange diffusion) to the steady-state hyperpolarization currents fi *all* our expts. in which these two parameters were measured on skins from the same anim

Strictly the correlation analysis ought to be based on maximum chloride conductance ersus exch diffusion flux. But, from the start, the expts. were not designed for this purpose, so conductance meas ments were not performed.

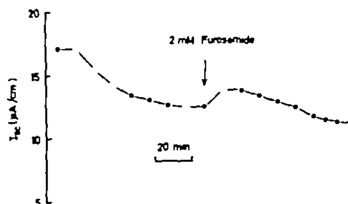


Fig 4 The effect short cir current of adding furosem (2 mM both sides) Averaged sh circuit current records from th expts. of Table V in wh chloride efflux measurements performed

urosemide inhibits most of the efflux of chloride under short circuit conditions, the remaining fraction is only slightly larger than that found when the skin is bathed on the outside with sulphate Ringer's. A similar effect has been reported by Bevis *et al.* (1974) on both influx and efflux of chloride across the frog skin (*Rana temporaria*). In skins from the same species, Natochin (1975) found that furosemide increases the transepithelial potential difference without affecting the short circuit current. The increase in potential difference was not seen with sulphate-Ringer's bathing the outside of the skin, so he concluded that furosemide acts on a chloride pathway. In our expts. furosemide was added to both sides of the skin, and in contrast to frog skin (Natochin 1975), the toad skin responded with a transient increase in short circuit current. The furosemide inhibition of chloride efflux under short circuit conditions as well as of clamping current under hyperpolarization, however, remained unchanged during the whole observation period after the onset of inhibition. The increase in short circuit current and inhibition of chloride transport, therefore, cannot be interrelated in a simple manner. With furosemide, a stimulation of short circuit current was also observed by Fillgraf *et al.* (1973), who likewise found that the amount of sodium in the skin exchanging with radioactive sodium in the outside medium was increased by furosemide (Aumann and Fillgraf 1975). They concluded that furosemide, when added to the outside, acts by increasing sodium permeability of the outer barrier and when added to the inside, that of the serosal membranes. The overall cellular sodium concentration however was not changed in the presence of the drug. Thus, it is unlikely that inhibition of chloride fluxes is caused by changes of cellular ion concentrations, and we conclude that furosemide interacts directly with chloride transport mechanisms. This conclusion is in accordance with that of Burg *et al.* 1973 who studied the effect of furosemide on various parts of the rabbit kidney tubule, and showed that the only effect of this diuretic is an inhibition of the transcellular chloride transport across the thick ascending limb of Henle's loop.

The effect of *phloretin* on sodium transport in epithelia has not been studied extensively. It has been reported to be ineffective when added to the outside solution of the toad bladder (*Bufo marinus*), and to reduce sodium transport by about 30%, when added to the inside bathing solution (Levine *et al.* 1973). In our experiments, an effect on short circuit current was not observed when the drug was added to both bathing solutions simultaneously. Thus, in the toad skin, it is clear that phloretin cannot affect the active sodium transport pathway. With this conclusion in mind it is interesting to note that phloretin was found to increase the transepithelial potential difference. This confirms our view (Bruus *et al.* 1976) that the cellular chloride conducting pathway is responsible for the low spontaneous potential difference observed across toad skins bathed on the outside with chloride-Ringer's.

In toad skin *acetazolamide* reduced chloride efflux under short circuit conditions as well as clamping current during hyperpolarization (Bruus *et al.* 1976). Under the experimental conditions used, the short circuit current was not affected. Also in frog skin, the short circuit current was unaffected by acetazolamide (Erlij 1971, Kristensen 1972). These results lead to the conclusion that the effects observed in toad skin are due to a direct interaction with chloride transport systems.

The mechanism of action of acetazolamide should be considered briefly. Since this drug

Discussion

The results presented very clearly show similar effects on chloride exchange diffusion and on transcellular anionic current as a result of drug application and anion substitution respectively. It is tempting, therefore, to consider whether the two types of chloride transfer are dependent upon a common membrane component. Such a possibility has been proposed for the chloride conductance and the chloride exchange diffusion in red cells (Vestergaard Bogind and Lassen 1974) based on pharmacological evidence; it was suggested again recently in the case of chloride transports in Ehrlich ascites tumor cells (Simonsen, Hoffmann and Sjøholm 1976). Before we discuss this hypothesis, however, it should be considered whether the observed effects on the two chloride transport mechanisms are direct ones or whether they are secondary, *i.e.* dependent on effects on other pathways, *e.g.* the active sodium transport.

Effect and specificity of ionic substitutions and of inhibitors

Anion substitutions and thiocyanate application significantly stimulate active sodium transport. The nature of these effects cannot be evaluated from the present study. In toad bladder Singer and Civan (1971) found that anions stimulate sodium transport with increasing efficiency according to the following order: Cl < Br < I < NO$_3$ < SCN. This is in agreement with our recordings of short circuit current during measurements of chloride effluxes. Although, SCN did not replace chloride, the effect on short circuit current was manifest. It is therefore likely that also in toad skin bromide, iodide, nitrate, and thiocyanate increase the sodium permeability of the outward facing membrane, as suggested by Singer and Civan (1971). Under conditions of an increased sodium transport it is unlikely that the cellular sodium concentration and, hence, the cellular chloride concentration decrease (Voûte and Hänni 1972). Therefore this cannot explain the reduction of chloride efflux seen with all other anions in the outside bathing solution. We conclude that bromide and iodide do not react with the exchange diffusion system as effectively as does chloride.

The clamping current measured under hyperpolarization with chloride Ringer's as outside medium is carried predominantly by an inward flux of chloride. The hyperpolarizing current is significantly reduced by substituting chloride in the outer bath with bromide or iodide. These responses are independent of whether the cellular sodium pathway operates or is blocked by amiloride. The most obvious explanation of these results therefore, is that the difference in halide currents reflects the abilities of these anions to enter and pass the conducting channels. Bearing in mind that the mobilities of chloride, bromide and iodide in water differ by less than 3 %, the observation of large differences in halide currents makes it probable that not the hydrated but the bare ions are the kinetic entities of the charge transfer process.

Brazy and Gunn (1976) showed that *furosemide* interacts primarily with the chloride transport mechanism in human red cells. However, the mechanism of action of furosemide in amphibian epithelia is not clarified, and the effect of this drug cannot *a priori* be considered to be due to a primary action on chloride transport mechanisms. In our

…nese two modes of chloride transfer, we suggest that the exchange process and the charge …ransferring process are both confined to the same type of membrane molecules which, in …ne state, serve chloride exchange diffusion and, in the other, conductive chloride transfer. … Because of the selectivity of the conducting pathway it can be supposed that the channels … specific sites for chloride binding. In the case of chloride exchange diffusion, one would …lso expect the membrane molecules involved to have binding sites for chloride. For exchange transport to occur these sites must be exposed alternatively to the two sides of the membrane, and since the chloride efflux is a saturable function of outside chloride concentration of the outer solution (Bruus *et al.* 1976), binding of chloride to the sites must be a prerequisite for the transfer process to occur. This may be achieved in many ways, which can not yet be distinguished from each other, at least not in a complicated epithelium. The range of possibilities covers the classical mobile carrier, a rotating carrier, conformational changes, and gate-like mechanisms. To change any of these systems from a non-conducting to a conducting state involves only that the system be transformed, so that binding of chloride is not a prerequisite for the respective molecular movements included in all models of exchange diffusion. In the language of the mobile carrier hypothesis, the chloride carrier can move chloride free across the membrane. Such a change may be conceived to be caused either directly by changes in clamping voltage, or by changes in concentration of ions interacting with *e.g.* a regulatory site. The present results do not allow any conclusion with respect to these problems. Neither is it possible to point out any reason for the quantitative differences observed with respect to the efficiency with which ion substitutions and inhibitors act on the two modes on chloride transport.

The correlation between the exchange diffusion flux measured under short circuit conditions and the outward clamping current obtained in the voltage range of full conductance activation does not necessarily imply a causal interdependence of these two variables. However, the correlation is in agreement with the hypothesis of a common molecular equipment of the two pathways. Therefore, the results of the pharmacological and the ionic specificity studies considered together with the result of the statistical analysis bring strong support to the hypothesis that the chloride exchange diffusion and the charge transferring transport of chloride take place via common membrane components.

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is known to inhibit carboanhydrase activity it has been suggested that its inhibitory effect what is assumed to be a Cl –HCO_3^- exchange in fish gill epithelium (Maetz and Garc Romeu 1964 Maetz 1971) and in amphibian skin (Garcia Romeu, Salibián and Pezza Hernández 1969 Erlij 1971) is due to a reduction of endogeneously produced HCO_3 significant carboanhydrase activity confined to the "flask cells" in the upper epidermis o *Rana pipiens* has been demonstrated (Rosen and Friedley 1973) The equality of outwa clamping current and inward transport of chloride rules out the possibility that a signific HCO outflux is associated with the conductive chloride influx through toad skin. Th conclusion is supported by the fact that addition of acetazolamide reduces the outwa clamping current and the inward flux of chloride to the same extent (Bruus *et al.* 1976). Th observation that chloride influx and outflux are equal in the toad skin clamped to reversed potential, together with the observation that the clamping current is now carrie by an inward flux of sodium, provide the evidence that HCO_3 does not significantl exchange with chloride (Bruus *et al.* 1976) Due to these findings it can hardly be assumed that a high level of carboanhydrase activity is required for these chloride transpor mechanisms. Therefore, we suggest that acetazolamide interferes directly with the pathways at the membrane level. A direct interaction of acetazolamide with a membrane component has also been suggested in the case of the neutral NaCl uptake across th mucosal membrane in rabbit ileum (Nellans, Frizzell and Schultz 1975) Likewise, the carbo- anhydrase inhibitor sulphonamide has been shown to interact directly with the carbo- anhydrase independent Cl Cl exchange in the membrane of ox red blood cells (Cousin and Motais 1976)

Although the drugs used may also affect the short circuit current (presumably the activ sodium transport) the above discussion leads to the conclusion that the results obtained ar caused by interactions between the drugs and the two chloride pathways.

The relation between the chloride conducting pathway and the chloride exchang pathway

In the preceding paper (Hvid Larsen and Kristensen 1977) it was shown that the chlorid conductance is a function of the transepithelial clamping voltage After a stepwise increase in clamping voltage, the conductance is time dependent and reaches its new steady state in the course of minutes. By comparing the results obtained with the predictions of a compute model of the two-membrane hypothesis, we came to the conclusion that the voltag dependence of the chloride conductance could not be explained solely by changes in membrane potentials and in intracellular chloride concentrations effectuating a "Goldman rectification" but that changes in chloride permeability must take place The increase in chloride permeability occurring after hyperpolarization could be a direct consequence of the change of a membrane voltage. Alternatively it could be caused by changes in the intracellular concentration of some ion interacting with a modifying site, thereby regulating the chloride permeability Our experiments do not enable us to distinguish between these two possibilities, but in both cases the increase in chloride permeability can be visual ized as formation or opening of "chloride channels" ("gating").

In this paper we have compared the exchange diffusion pathway to the pathway allowing conductive transfer of chloride ions. In order to account for the striking similarity

…ese two modes of chloride transfer we suggest that the exchange process and the charge transferring process are both confined to the same type of membrane molecules which, in one state, serve chloride exchange diffusion and, in the other conductive chloride transfer. Because of the selectivity of the conducting pathway it can be supposed that the channels ... specific sites for chloride binding. In the case of chloride exchange diffusion, one would also expect the membrane molecules involved to have binding sites for chloride. For exchange transport to occur these sites must be exposed alternatively to the two sides of the membrane, and since the chloride efflux is a saturable function of outside chloride concentration of the outer solution (Brown *et al.* 1976), binding of chloride to the sites must be a prerequisite for the transfer process to occur. This may be achieved in many ways, which can not yet be distinguished from each other at least not in a complicated epithelium. The range of possibilities covers the classical mobile carrier a rotating carrier conformational changes, and gate-like mechanisms. To change any of these systems from a non-conducting to a conducting state involves only that the system be transformed, so that binding of chloride is not a prerequisite for the respective molecular movements included in all models of exchange diffusion. In the language of the mobile carrier hypothesis the chloride carrier can move chloride free across the membrane. Such a change may be conceived to be caused either directly by changes in clamping voltage, or by changes in concentration of ions interacting with a regulatory site. The present results do not allow any conclusion with respect to these problems. Neither is it possible to point out any reason for the quantitative differences observed with respect to the efficiency with which ion substitutions and inhibitors act on the two modes on chloride transport.

The correlation between the exchange diffusion flux measured under short circuit conditions and the outward clamping current obtained in the voltage range of full conductance activation does not necessarily imply a causal interdependence of these two variables. However the correlation is in agreement with the hypothesis of a common molecular equipment of the two pathways. Therefore, the results of the pharmacological and the ionic specificity studies considered together with the result of the statistical analysis bring strong support to the hypothesis that the chloride exchange diffusion and the charge transferring transport of chloride take place via common membrane components.

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is known to inhibit carboanhydrase activity It has been suggested that its inhibitory effect what is assumed to be a Cl –HCO_3^- exchange in fish gill epithelium (Maetz and Gar Romeu 1964 Maetz 1971) and in amphibian skin (Garcia Romeu, Salibián and Pez Hernández 1969 Erlij 1971) is due to a reduction of endogeneously produced HCO significant carboanhydrase activity confined to the "flask cells" in the upper epiderm *Rana pipiens* has been demonstrated (Rosen and Friedley 1973). The equality of outw clamping current and inward transport of chloride rules out the possibility that a signific HCO outflux is associated with the conductive chloride influx through toad skin. T conclusion is supported by the fact that addition of acetazolamide reduces the outw clamping current and the inward flux of chloride to the same extent (Bruus *et al* 1976) T observation that chloride influx and outflux are equal in the toad skin clamped reversed potential together with the observation that the clamping current is now car by an inward flux of sodium provide the evidence that HCO does not signific exchange with chloride (Bruus *et al* 1976) Due to these findings it can hardly be assu that a high level of carboanhydrase activity is required for these chloride transp mechanisms Therefore, we suggest that acetazolamide interferes directly with th pathways at the membrane level A direct interaction of acetazolamide with a membr component has also been suggested in the case of the neutral NaCl uptake across mucosal membrane in rabbit ileum (Nellans, Frizzell and Schultz 1975) Likewise, the car anhydrase inhibitor sulphonamide has been shown to interact directly with the car anhydrase independent Cl Cl exchange in the membrane of ox red blood cells (Co and Motais 1976)

Although the drugs used may also affect the short circuit current (presumably the ac sodium transport) the above discussion leads to the conclusion that the results obtained caused by interactions between the drugs and the two chloride pathways.

The relation between the chloride conducting pathway and the chloride exchang pathway

In the preceding paper (Hviid Larsen and Kristensen 1977) it was shown that the chlo conductance is a function of the transepithelial clamping voltage After a stepwise increas clamping voltage, the conductance is time dependent and reaches its new steady stat the course of minutes. By comparing the results obtained with the predictions of a comp model of the two-membrane hypothesis, we came to the conclusion that the volt dependence of the chloride conductance could not be explained solely by changes membrane potentials and in intracellular chloride concentrations effectuating a "Goldm rectification" but that changes in chloride permeability must take place. The increas chloride permeability occurring after hyperpolarization could be a direct consequence the change of a membrane voltage. Alternatively it could be caused by changes in intracellular concentration of some ion interacting with a modifying site, thereby regulat the chloride permeability Our experiments do not enable us to distinguish betw these two possibilities, but in both cases the increase in chloride permeability can be visu ized as formation or opening of "chloride channels" ("gating")

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From the Department of Neurology, University of Helsinki, Finland

On the neuromuscular effects of pindolol and sotalol in the rat

By

ANDREJO LARSEN

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Abstract

LARSEN, A. *On the neuromuscular effects of pindolol and sotalol in the rat.* Acta physiol. scand. 1978. *102*. 35–40.

The effects of pindolol and sotalol on neuromuscular transmission were tested using intracellular micro-electrode recording of resting membrane potentials, miniature end-plate potentials and end-plate potentials, and recording of muscle contractions upon nerve stimulation of rat phrenic nerve-hemidiaphragm preparations. Both pindolol and sotalol reduced the amplitudes of miniature end-plate potentials and end-plate potentials in a dose-dependent manner without significantly affecting their time-courses. Pindolol, but not sotalol, also increased the frequency of the miniature end-plate potentials and decreased the number of acetylcholine quanta released by nerve impulses. Neither of the drugs significantly affected resting membrane potentials of the muscle fibres or excitability of the motor nerve to electric stimulation.

Key words: neuromuscular transmission, pindolol, sotalol

The beta-blocking agent propranolol inhibits neuromuscular transmission both by exerting a postsynaptic curare-like effect (Lilleheil and Røed 1971, Werman and Wislicki 1971, Larsen and Teräväinen 1977) and by decreasing the number of transmitter quanta released from motor nerve endings by a nerve impulse (Larsen and Teräväinen 1977).

The possible effects of other beta-blocking drugs on neuromuscular transmission have not been studied with intracellular recording methods. The present study was made in order to compare pindolol and sotalol, which differ from each other in their pharmacological properties. Pindolol has an intrinsic sympathomimetic activity and a membrane stabilizing activity (Levy 1971), whereas sotalol is devoid of both of these effects (Lish *et al.* 1965).

Methods

A total of 69 Sprague-Dawley rats, weighing 190 to 270 g, were used in the experiments. The animals were anesthetized with ether and decapitated. The left diaphragm muscle and the phrenic nerve were dissected and prepared further in continuously oxygenated bathing solution. The phrenic nerve-hemidiaphragm preparations were placed in a recording chamber with a fluid volume of 4 ml. The bathing solution had the following composition (in mmol/l): NaCl 135, $NaHCO_3$ 16.5, Na_2HPO_4 1, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1 and glucose 11. It was continuously equilibrated with a mixture of 95 % oxygen and 5 % CO_2 and had a pH of 7.35–7.40. The fluid in the recording chamber was replaced at a constant rate of 400 ml

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From the Department of Neurology University of Helsinki, Finland

On the neuromuscular effects of pindolol and sotalol in the rat

By

ANDERS LARSEN

Received 30 May 1977

Abstract

LARSEN, A. *On the neuromuscular effects of pindolol and sotalol in the rat* Acta physiol. scand. 1978. *102* 35–40.

The effects of pindolol and sotalol on neuromuscular transmission were tested using intracellular microelectrode recording of resting membrane potentials, miniature end-plate potentials and end-plate potentials, and recording of muscle contractions upon nerve stimulation of rat phrenic nerve-hemidiaphragm preparations. Both pindolol and sotalol reduced the amplitudes of miniature end-plate potentials and end-plate potentials in dose-dependent manner without significantly affecting their time-courses. Pindolol, but not sotalol, also increased the frequency of the miniature end-plate potentials and decreased the number of acetylcholine quanta released by nerve impulses. Neither of the drugs significantly affected resting membrane potentials of the muscle fibres or excitability of the motor nerve to electric stimulation.

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The beta-blocking agent propranolol inhibits neuromuscular transmission both by exerting postsynaptic curare-like effect (Usubiaga and Roed 1971 Werman and Wislicki 1971 Larsen and Teräväinen 1977) and by decreasing the number of transmitter quanta released from motor nerve endings by nerve impulse (Larsen and Teräväinen 1977).

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Methods

A total of 69 Sprague Dawley rats, weighing 190 to 270 g, were used in the experiments. The animals were anaesthetized with ether and decapitated. The left diaphragm muscle and the phrenic nerve were dissected and prepared further in continuously oxygenated bathing solution. The phrenic nerve-hemidiaphragm preparations were placed in recording chamber with fluid volume of 4 ml. The bathing solution had the following composition (in mmol/l): NaCl 135, $NaHCO_3$ 16.3, Na_2HPO_4 1, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, and glucose 11. It was continuously equilibrated with mixture of 95% oxygen and 5 CO_2 and had pH of 7.35-7.40. The fluid in the recording chamber was replaced at constant rate of 400 ml

per hour The drugs were dissolved in the bathing solution and introduced into the recording chamb from another reservoir The drugs used were pindolol (Sandoz Ltd., Switzerland) mixed with tartaric ac 2 1 and sotalol chlorhydrate (Lääke Oy Finland). The preparations were usually allowed to equilibra for 30 min in the drug-free solution (control solution) or in the solution containing either of the drug (pindolol and sotalol solution) both before th recording was begun and after the solutions were changed

Intracellular recordi g of resting membrane potentials (RMP), miniature end-plate potentials (MEPP and end-plate potentials (EPP) was made with glass capillary microelectrodes filled with 3 M KCl an selected for resistances between 5 and 15 megaohms. A suction electrode was used to stimulate the phren nerve with a Grass S-8 stimulator with an SIU 4678 stimulus isolation unit. The potentials were amplifie with a *Mentor N-950 preamplifier* a*nd displayed on a Tektronix 5115 oscilloscope. The traces were photo* graphed from the storage oscilloscope screen and measured from magnified negatives.

All the recordings were made at a bath temperature of 23°C, and only those f bres with an initial RM of at least −65 mV and which did not depolarize more than 5 mV duri g the expts. were used MEPP with a rise time of less than 1 ms were included in the statistics. The MEPP amplitudes were corrected fo differences in the RMPs of individual fibres using −75 mV as the standard RMP (Katz and Thesleff 195 About 50 MEPPs were counted from each f bre

In studying EPPS the phrenic nerve was stimulated with supramaximal current pulses 0 02 ms i duration nd the amplitudes of the EPPs were reduced below the threshold for action potentials either b increasing the concentration of Mg^{++} with a concomitant reduction of Ca^{++} concentration (see Result or by adding $7–8 \times 10^{-}$ g/ml d-tubocurarine to the solution.

The quantum content (m) in high-Mg expts. was calculated from bout 120 successive EPPs from eac f bre as m = ln (number of impulses/number f failures) and as m = mean EPP amplitude/mean MEP amplitude (del Castillo and Katz 1954), fter correcting the amplitudes for nonlinearity of respon (M rtin 1955). Only those fibres which gave at least 90% correlation between the two estimates we included in the statistics in which the exact value of the latter formula was used. In the expts. where th quantum content was so high that no failures occurred only the latter formula was used. In order t estimate the size of the qua ta and the mean quantum content in the curarized preparations, 60 successi EPPs were recorded from each f bre and the EPP amplitudes were corrected for nonlinearity of respon and *differences in RMPs using the formula given by Elmqvist and Quastel (1965)· EPP'* (corrected EPP) EPP/(RMP − 15 mV − EPP) The qua tum size (q) was calculated as q = (variance of EPP')/(mean EPP (Elmqvist and Quastel 1965) and the quantum content as m = (mean EPP')²/(variance of EPP's), / m⁻¹ coefficient of variati n f EPP' (Martin 1955). To convert back to mV values, q was multiplied b (RMP − 15 mV), (Elmqvist and Quastel 1965) The stimulation frequency in all quantum content expt was 1 Hz.

To study the excitability characteristics of the motor nerve (strength—duration relationship), the phren nerve was stimulated with pulses of varying strengths nd d rat ons and the contractions of the diaphrag were recorded electrically using the strain-gauge principle.

The hemidiaphragm was fi ed with insect pins to the bottom of the recordi g chamber only at th costal margin. An elastic steel blade w th Philips PR 9833K/OGSE strain-ga ges cemented on both sid was attached by a small hook to the free membraneous side f the diaphragm, and the contractions recorde with the strain-ga ges were amplified with Tektroni 12 preamplifier and fed to a Tektronix 511 storage oscilloscope Student s t-test was used to determine the statistical significance of the differenc bet een the means.

Results

The mean of the resting membrane potentials (RMP) of 90 fibres from preparation treated with pindolol (10 to 100 mg/l) was 77.2 ± 0 8 mV (mean ± S E.) and that of 92 fibre recorded in sotalol solution (10 to 100 mg/l) was 77 1 ± 0 7 mV Neither of these value differed significantly from the control value of 77 4 ± 0 7 mV (mean ± S E.) obtained from 90 fibres.

The effect of pindolol and sotalol on the amplitudes of the miniature end-plat potentials (MEPP) is shown in Fig. 1 Both drugs caused a dose-dependent reduction in th MEPP amplitudes.

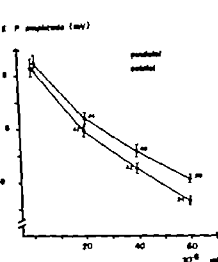


Fig. 1

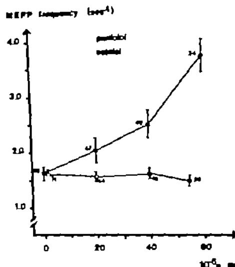


Fig. 2

Fig. 1. The figure illustrates the effects of pindolol and sotalol on the miniature end-plate potential (MEPP) amplitudes. A dose-dependent reduction is caused by both drugs. The points are the mean values of the number of fibres indicated by the small numbers adjacent to the points. Bars represent ± S.E. Ordinate: amplitude of the miniature end-plate potentials in mV. Abscissa: the concentration of the drugs.

Fig. 2. The effect of pindolol and sotalol on the frequency of the spontaneous release of transmitter (mean and S.E.). Pindolol increases the frequency, the effect being dependent of the concentration of the drug. No significant effect was observed with sotalol. Ordinate: the frequency of the miniature end-plate potentials (MEPP). Abscissa: the concentration of the drugs. The small numbers are the numbers of fibres studied.

The effect of the drugs began to manifest itself a few minutes after the beginning of the perfusion with either of the test solutions. The full strength of the effect was reached in 20 min. The drugs did not differ in this respect.

Fig. shows the effect of the drugs on the frequency of the occurrence of MEPPs. Pindolol caused an increase in the frequency depending on the concentration of the drug, but sotalol had no significant effect. After washing the preparation for 30 min with the control solution, both the MEPP amplitudes and frequency were comparable to the controls, within an accuracy of at least 95%. The rise-time of the MEPPs recorded from 20 fibres in 40 mg/l pindolol solution was 0.52 0.02 ms (mean ± S.E.), and that of 22 fibres in 40 mg/l sotalol solution 0.51 0.02 ms (mean S.E.). Compared with the control value of 0.52 0.0_ ms (mean S.E.), recorded from 26 fibres, no significant difference existed.

Both pindolol and sotalol caused a dose-dependent reduction of the end-plate potential (EPP) amplitudes in curarized preparations (Fig. 3). The effect was similar to that observed in the MEPP amplitude: the maximal effect was reached in 20 min and was reversible. The effect of pindolol was stronger than that of sotalol, the approximate concentrations needed to produce a 1/e-fold reduction of EPP amplitude compared with the control being 30 mg/l of pindolol and 45 mg/l of sotalol.

Both pindolol and sotalol (40 mg/l) significantly reduced the size of the quanta in

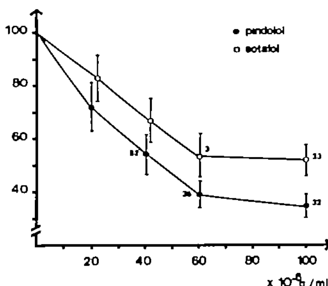


Fig. 3 The figure illustrates the dose-dependent reduction of the end-plate potential amplitude with pindolol and sotalol as percentage of the control. Mean and S.E. Ordinate the mean amplitude of end plate potentials (EPP) percentage of the control. Abscissa the concentration of the drugs. The small numbers indicate the numbers of fibres studied.

curarized preparations (Table I) Neither of the drugs had significant effects on the time courses of the EPPs (Table I)

The effect of the drugs on the number of transmitter quanta released from the motor nerve endings by nerve stimulation was analyzed both in high-Mg-blocked (Mg^{++} 23 mmol/l and Ca 3 mmol/l Mg 9 mmol/l and Ca 1 mmol/l) and in curarized preparations. Pindolol significantly reduced the mean quantum content in all three solutions tested (Table II) The effect was strongest in curarized preparations where the transmitter release was also greatest No significant effect of sotalol on the transmitter release was observed (Table II)

The excitability of the motor nerve to electrical stimulation was studied by stimulating the phrenic nerve at a rate of one pulse per second and determining the weakest current strength sufficient to produce constant twitches of the diaphragm muscle at a fixed stimulus duration No significant differences in the excitability of the nerve were observed either

TABLE I The effect of pindolol and sotalol on end-plat potentials in curarized muscle (Mean ± S E)

	Amplitude (% of control)	Rise time (ms)	H lf time (ms)	Qua tum size (mV)
Control	100 (82)[a]	1.04 ± 0 04 (15)	1.31 ± 0 06 (15)	0 074 ± 0 007 (24)
Pindolol (40 mg/l)	54.2 ± 7 1[b] (52)	1 03 ± 0 04 (15)	1.29 ± 0 06 (15)	0 013 ± 0 001[b] (24)
Sotalol (40 mg/l)	66.3 ± 8 [b] (48)	1.03 ± 0 04 (15)	1.31 ± 0.05 (15)	0 015 ± 0 01[b] (26)

N of end-plates tudied
[b] S.gn ant at the level $p < 0\,001$ compared with the control.

Table II The effect of pindolol and sotalol on the quantum content of end-plate potentials (mean ± S.E.). A shows the quantum content recorded in solution containing 7 10^{-5} g/ml d-tubocurarine, B in solution containing 23 mmol/l Mg^{++} and 3 mmol/l Ca^{++} and C in solution containing 9 mmol/l Mg^{++} and 1 mmol/l Ca^{++}. The stimulus frequency was 1 Hz

	A	B	C
Control	103.1 ± 4.7 (24)[a]	9.23 ± 0.50 (18)	1.31 ± 0.04 (28)
Pindolol (40 mg/l)	73.7 ± 5.6 (24)	7.32 ± 0.44[b] (18)	1.17 ± 0.03[c] (29)
Sotalol (40 mg/l)	101.9 ± 4.1 (24)	9.29 ± 0.52 (19)	1.27 ± 0.04 (28)

Number of end-plates studied.
Significant at the level p 0.005 compared with the control.
Significant at the level p 0.01 compared with the control.

n the presence of pindolol (40 to 100 mg/l) or sotalol (40 to 100 mg/l) using pulse durations of 0.1 to 0.10 ms. The threshold current strengths for both drugs were kept within ±2% of the control values.

Discussion

It is evident from the present results that both pindolol and sotalol inhibit neuromuscular transmission by reducing the amplitudes of MEPPs and EPPs in a dose-dependent manner. Pindolol also reduces the number of acetylcholine quanta released from the motor nerve endings by nerve impulses, even though it slightly increased the frequency of the spontaneous release of MEPPs. Neither of the drugs seems to affect the rise-time or the half decay time of MEPPs and EPPs or the excitability of the motor nerve to electrical stimulation. That the reduction of the amplitudes of the MEPPs and EPPs is postsynaptic, and not due to a diminished amount of acetylcholine within quanta, or a depletion of the transmitter stores, is suggested by the rapidity and reversibility of action of the drugs. As it is dose-dependent and not combined with any changes in the time courses of the potentials, the postsynaptic action of the drugs is likely to be competitive inhibition resembling that of the curare effect.

In addition to the postsynaptic actions, pindolol had presynaptic effects on the transmitter release: It increased the frequency of MEPPs at the resting stage and diminished the release induced by nerve impulses. The first type of action of pindolol may be due to the intrinsic sympathomimetic activity of the drug similar to that of adrenaline, causing an increase in the MEPP frequency (Kuba 1970). Judging from the significances of the differences compared with the control, the postsynaptic effects of pindolol seem to be quantitatively more important than the presynaptic actions in the impulse transmission at the motor end-plate.

The depression of the quantal release of the transmitter upon nerve stimulation was most marked when the quantum content was high. It diminished proportionally when the mean quantum content was reduced by changing the ratio of Mg^{++} and Ca^{++} ions. This

seems to exclude the possibility that pindolol blocks a constant part of the population of transmitter quanta available for release. The reduction by pindolol of the number of acetylcholine quanta released may be due either to a reduced depolarization of the motor nerve endings or to a diminished probability of release in response to depolarization, or both.

Compared with the previously reported effects of propranolol on the neuromuscular transmission (Larsen and Terävăinen 1977) the actions of pindolol and sotalol at the postsynaptic site were qualitatively similar At the presynaptic site, however important differences were observed Propranolol and pindolol, which have a membrane-stabilizing effect (Morales-Aguilerá and Vaughan Williams 1965 Levy 1971), were observed to depress the stimulus-induced transmitter release. Sotalol, which is devoid of any marked membrane effect (Lish *et al* 1965), had no significant effects on the transmitter release. On the other hand pindolol, which is the only one of these drugs with an intrinsic sympathomimetic activity, increased spontaneous transmitter release.

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From the Department of Physiology University of Göteborg, Sweden

Cardiovascular reactivity and design in rats with experimental "neurogenic hypertension"

By

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Abstract

Jones, J. V. and M. Hallbäck. *Cardiovascular reactivity and design in rats with experimental neurogenic hypertension*. Acta physiol. scand. 1978. *102*. 41–49.

The arterial baroreceptors were denervated in 20 normotensive Wistar rats. 2 1/2 months after the operation their cardiovascular responses to "mental stress" were compared to those of matched control rats (NCR). At the time of investigation the blood pressure of the baroreceptor denervated rats (BDR) was increased some 15 per cent above that of the control group. There was, however, no difference in response to mental stress between groups, if anything the BDR responded with less pronounced tachycardia. Two months subsequent to the stress-test the mean blood pressure of the BDR was still significantly above normal levels, but the BDR did not exhibit structural cardiovascular adaptation, which has been documented in most types of stable hypertension as response to the increased mean blood pressure. The results imply that hypertension induced by baroreceptor denervation is not of a stable and persistent type in which case structural changes in the cardiovascular system would have developed within the observation period (approximately 4 months). Emotional stimuli, however, do not seem to contribute to periodic blood pressure increases at the time of measurement, as earlier suggested.

Following their description by Hering in 1923 the systemic arterial baroreceptors were extensively investigated as a possible source of the aetiology of essential hypertension in man. It soon became apparent that section of the buffer nerves did not produce a model of essential hypertension but rather that a fluctuating blood pressure elevation and tachycardia resulted from this procedure (*cf.* Heymans and Neil 1958). Recently Cowley, Liard and Guyton (1973) have challenged even this assertion and have postulated that the arterial baroreceptors have little or no role to play in determining mean blood pressure integrated over the 4 h, but instead operate in such a manner as to oppose acute changes in blood pressure around the mean, at least in the dog. In other words, the baroreceptors do indeed function as buffer nerves in such a way that they not only minimise rises in blood pressure but they also lessen falls in blood pressure. According to these authors, the mean blood pressure itself is determined by other factors, possibly renal in origin.

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seems to exclude the possibility that pindolol blocks a constant part of the population of transmitter quanta available for release The reduction by pindolol of the number of acetylcholine quanta released may be due either to a reduced depolarization of the motor nerve endings or to a diminished probability of release in response to depolarization, or both.

Compared with the previously reported effects of propranolol on the neuromuscular transmission (Larsen and Terävälnen 1977) the actions of pindolol and sotalol at the postsynaptic site were qualitatively similar At the presynaptic site, however important differences were observed Propranolol and pindolol which have a membrane-stabilizing effect (Morales-Aguilerá and Vaughan Williams 1965 Levy 1971) were observed to depress the stimulus-induced transmitter release. Sotalol which is devoid of any marked membrane effect (Lish *et al* 1965) had no significant effects on the transmitter release. On the other hand, pindolol which is the only one of these drugs with an intrinsic sympathomimetic activity, increased spontaneous transmitter release.

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allowed to recover. In 5 animals a catheter was placed in the caudal artery and the blood pressure recorded throughout the operation which usually took from 30–40 min to perform. In all 5 animals heart rate and blood pressure increased as a result of these manoeuvres. The rats were left for 2–2 1/2 months and then subjected to a standardized "mental stress" test (cf. Hallbäck and Folkow 1974).

On the day of the "mental stress" test the animals were anesthetized briefly with ether and a cannula for recording blood pressure and heart rate was inserted into the tail artery. The rats were always studied in pairs with one baroreceptor denervated rat (BDR) and one normotensive control rat (NCR). Local anesthetic was infiltrated round the base of the tail to minimize irritation from the site of insertion of the cannula.

The rats were then placed in a specially designed box with two identical chambers. Details of the stressing procedure have been published previously (Hallbäck and Folkow 1974). The stress stimuli consisted of the repeated application (30 s) of either disturbing sound or vibration, after an initial acclimatization to the new environment of one hour's duration. 15 min recovery periods between the two types of stress were allowed. Sound stress always preceded stress produced by vibration. Finally the animals were left for another period of 1 h so that the blood pressure could be monitored for a further period during which the variability in blood pressure was determined. This was done by counting each deviation greater than 5 mmHg from the resting blood pressure that occurred during this hour. It was also of interest to see whether a longer period of acclimatization to the new environment would cause a further stabilization of blood pressure in BDR.

At the end of this additional period of observation and during stable blood pressure and heart rate conditions, 1.0 µg/kg of angiotensin was injected intra-arterially. The subsequent effect of the acute rise in blood pressure on the heart rate was then recorded as a test of baroreceptor efficiency in comparison with the normotensive controls. After terminating the expt. the tail artery cannula was removed under brief ether anesthesia and the animals returned to the animal house for a further period of 1 1/2 months before a study of cardiac and vascular design was made by means of a hindquarter perfusion technique. 15 NCR and 15 out of the original BDR were included in the study. These were selected on the basis of the BDR having the highest blood pressures at the time of stress testing.

The presence of an elevated blood pressure (at least during the period of measurement) was confirmed also at the time of the hindquarter perfusion studies by insertion of a cannula into the tail artery under brief ether anesthesia and then allowing the animal to regain consciousness so that awake heart rate and blood pressure could be determined.

Pairs of rats that had been used together during the stress procedure (i.e. one NCR and one BDR) were again studied together using a paired hindquarter perfusion technique (cf. Folkow et al. 1970). Oxygenated Tyrode solution containing 4% dextran as colloid substitute was used as a perfusate. In all other respects the technique was as used by Folkow et al. 1970 and the construction of the resistance curves characterizing the individual vascular beds was as described by these authors. Briefly, during maximal dilatation, induced by repeated data injections of papaverine, pressure flow curves were constructed for each pair of hindquarters from the pressure-flow recordings. The pressure responses during constant flow (i.e. resistance responses) to graded doses of noradrenaline (NA) were obtained in each pair of rats. Resistance curves were then constructed for each pair of animals with log NA dose/ml perfusate on the abscissa and perfusion pressure in mmHg on the ordinate. From these curves could be calculated the resistance at maximum dilatation, the threshold dose of noradrenaline which is defined as the noradrenaline concentration required to raise resistance 25 per cent above that at maximal dilatation, the steepness of the curve as well as the maximum pressor response which was induced by large doses of vasopressin (10 IU) or barium chloride, 150 mg. The mean ± S.E. for these points were calculated for both groups of rats and the differences between the BDR group compared to the NCR group evaluated by means of the pairing design t-test.

The hearts were removed from all the animals and the left ventricles dissected free and weighed. Then the mean left ventricular weight was calculated as was the percentage left ventricular weight/body weight and mean values of the two groups compared.

Results

In a preliminary test four rats died during the operation or immediately thereafter. With a slight modification of the operation technique 20 operated rats all survived the operation. 15 of these BDR were studied further on the arbitrary basis of their higher blood pressures as measured after one hour of acclimatization in the stress box but before the first period of

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The present study was undertaken to try to clarify some of these apparently contradic results. In order to explore if baroreceptor denervation causes an increased cardiovasc reactivity to alerting stimuli rats were exposed to a standardized "mental" stress 2 mon after denervation of the arterial baroreceptors A further 2 months later the rats w investigated by means of a hemodynamic analysis to explore if structural changes in cardiovascular system had occurred as an adaptive response to an increased mean arte pressure, as previously has been shown in other types of chronic hypertension (*cf* Folk *et al* 1973)

Methods

20 male Wistar rats weighing pproximately 200 g nd ged 7 week were subjected to slight modi tion of Krieger s (1964) "standard technique for the production of neurogenic hypertension in the The proced re was briefly that of section and removal of a length f both superior laryngeal nerves both cervical sympathetic trunk under ether anesthesia. This resulted i denervation of the aortic b receptors. The carotid sinuses were carefully cleared of all connective tissue nd then pai ted th l phenol in lcohol as extensi ely as possibly resulting in their denervation also. The incision in the was sutured and infiltrated with local nesthetic after which the ether wa disconti ued and the ani

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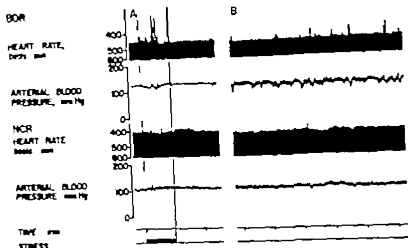
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The present study was undertaken to try to clarify some of these apparently contradictory results. In order to explore if baroreceptor denervation causes an increased cardiovascular reactivity to alerting stimuli rats were exposed to a standardized "mental" stress months after denervation of the arterial baroreceptors. A further 2 months later the rats were investigated by means of a hemodynamic analysis to explore if structural changes in the cardiovascular system had occurred as an adaptive response to an increased mean arterial pressure, as previously has been shown in other types of chronic hypertension (*cf* Folkow *et al* 1973)

Methods

20 male Wistar rats weighing approximately [illegible]00 g and aged 7 weeks were subjected to a slight modification of Krieger (1964) standard technique for the production of neurogenic hypertension in the rat. The procedure was briefly that of section and removal of length of both superior laryngeal nerves and both cervical sympathetic trunk under ether anesthesia. This resulted in denervation of the aortic baroreceptors. The carotid sinuses were carefully cleared of all connective tissue and then painted with 10% phenol in alcohol as extensively as possibly resulting in their denervation also The incision the neck was sutured and infiltrated with local anesthetic after which the ether was discontinued and the animals



1 Part A shows the actual heart rate and blood pressure traces from one baroreceptor denervated rat (BDR, upper two recordings) and from one matched normotensive control rat (NCR, lower two recordings) which were simultaneously investigated in standardized "stress-test" The stimulus applied was in this exposure to violent vibrations. Part B is continuous 5 min recording of blood pressure and heart rate of the two rats Note the greater lability in blood pressure in the BDR.

Variability in basal blood pressure was decidedly more marked in BDR than in NCR. During the 1 h observation following the periods of stress blood pressure deviated more than 5 mmHg from baseline 240 ± 21 times in BDR while the equivalent figure for NCR was 142 ± 19 (p 0.001). The great majority of these deviations from resting pressure were, however *falls* in blood pressure (see Fig. 1).

As a test of baroreceptor function the reflexogenic bradycardia in response to an angiotensin-induced blood pressure increase was investigated. During the rapid initial blood pressure rise, an increase of 25 mmHg caused the heart rate to fall 25 ± 4 beats per min in the NCR No change at all, or 0.1 ± 5 beats per min, was observed in the BDR indicating that BDR were efficiently deprived of their arterial baroreceptor control.

At the time of the stress test the body weight of the BDR was 333 ± 6 g and that of the NCR was 343 ± 3 g and hence there was no difference in body weight. 6 weeks elapsed until the hemodynamic analyses were performed during which time the NCR increased in weight by 58 ± 5 g while the BDR only gained 34 ± 4 g. These differences in weight gain were significantly different (p 0.01) and, therefore, the NCR were significantly heavier (399 ± 7 g) than the BDR (370 ± 8) when the hemodynamic analysis was performed ($p < 0.01$)

The hemodynamic analyses were performed on 15 pairs of BDR and NCR. During simultaneous perfusion of the two hindquarter vascular beds in each pair there was no difference between BDR and NCR in resistance during maximal vasodilatation, *i.e.* when all vascular smooth muscle activity was abolished (see Table I). Sensitivity to noradrenaline was equal in the two groups as judged by equal threshold doses. The slope of the resistance curves of the two groups did not differ substantially and no difference in maximal pressor response was observed between the two groups (see Table I). Thus, as judged by these para

TABLE I Gives the mean values ± S E. for mean arterial blood pressure and heart rate for 15 baroreceptor denervated rats (BDR) and for 15 normotensive control rats (NCR) at the time of "stress-test". Furthermore from the hemodynamic analysis are presented the resistance at maximal vasodilation at a flow of 20 ml/min 100 g, the steepness of the resistance curve and the maximal pressor response. Left ventricular weight in per cent of body weight is also presented for the two groups. Levels of significant difference between groups are also given.

	Mean arterial blood pressure, mmHg	Heart rate, beats/min	PRU_{100} at max. dil. Q = 20 ml/min 100 g	Tangent of the angle of the resistance curve	Maximal pressor response mmHg	Left ventricular weight in per cent of body weight
BDR n = 15	126 ± 2	419 ± 7	2.4 ± 0.1	2.5 ± 0.3	219 ± 11	0.177 ± 0.004
NCR n = 15	111 ± 2	371 ± 8	2.5 ± 0.1	2.6 ± 0.4	209 ± 14	0.174 ± 0.004
Significance p <	0.001	0.001	0.5	1.0	0.5	0.5

stress (mean arterial blood pressure 126 ± 2 mmHg, see Table I) These 15 rats were considered to have "neurogenic hypertension" Mean arterial blood pressure in the control *group of rats was 111 ± 2 mmHg, which was significantly lower than that of the BDR* ($p < 0.001$) Thus the BDR had a mean blood pressure increase of 15 mmHg or 14% over the NCR at the time of recording. Just prior to the hindquarter perfusion study the BDR had a mean arterial blood pressure of 126 ± 2 mmHg and their matched NCR 107 ± 3 mmHg before anesthesia was given. Neither of these values, recorded in the awake rats, had changed significantly when compared to the same 15 pairs at the time of stressing. Mean arterial blood pressure after administration of nembutal but before the operative procedures were started were 125 ± 2 in BDR and 104 ± 3 in the NCR. There was hence no significant difference in mean blood pressure in either group after the barbiturate had been applied.

"Basal" values of blood pressure and heart rate were obtained in the awake rats after one hour s accomodation in the stress box in a semi-dark, soundproof room At this time the rats had recovered from the superficial ether anesthesia required for insertion of the tail artery cannula The mean changes in heart rate and blood pressure during the 30 s of stress and also during the succeeding 30 post stress seconds were obtained in the following way The changes in heart rate and blood pressure from prestimulatory value were calculated for each third second in each rat, after which the difference in response between the paired BDR and NCR at each third second interval was calculated The mean of these paired differences, and the standard errors of the mean differences, were then calculated as well as the significance differences in response (*cf* Hallbäck and Folkow 1974) A significant difference in total response was considered to be present when 6 out of the 10 observations during stress stimuli or 12 of the total 20 observations, differ above 95% probability

In this way it was found that the neurogenically hypertensive rats responded with less pronounced tachycardia than did the NCR This may be due to the higher resting heart rates in the BDR. However both groups preferentially responded with tachycardia in the stress period so that there was no difference between the BDR and NCR as to the direction of response during the stress stimulus. The blood pressure responses during stress were of equal magnitude in the two groups.

spontaneously hypertensive rat (MHS) showed a good correlation between the degree of left ventricular hypertrophy and the elevation of mean arterial blood pressure even though arterial blood pressure in these MHS was only elevated some 15 per cent (Hallbäck *et al.* 1976), as in the present BDR at the time of measurement. In rats, the structural adaptation of the left heart and precapillary resistance vessels is a rapid process being practically complete within 2–3 weeks of hypertension, when induced by renal artery obstruction (Lundgren *et al.* 1974). The presently used BDR were denervated for between 3–4 months. There more structural changes should have been detectable within this time scale in case the measured increase in mean arterial pressure of 15 per cent really represented the average increase throughout the 24-h during these 4 months. It would seem therefore that the mean blood pressure as here recorded in the awake animals, either before stressing or just prior to a hemodynamic analysis, is not a true reflection of the average pressure level over 24 h. The absence of the structural changes within the cardiovascular high-pressure compartments in the present BDR model strongly suggests that their average level of mean arterial pressure is not elevated over 24 h.

Following baroreceptor denervation in dogs Cowley *et al.* (1973) found wide swings in blood pressure thought to be associated with alerting stimuli. In particular the entry of the dog attendants into the room caused tachycardia and acute rises in blood pressure. After a few months, these rapid swings in pressure were less marked but still considerably more pronounced than in control dogs. Many such oscillations in the pressure occurred for apparently no external reason, *i.e.* without obvious alerting stimuli though there might of course, have been such stimuli that were experienced only by the animals and not by the experimentator. We observed similar more frequent pressure oscillations in BDR compared to NCR and many of these occurred when the animal appeared to be peaceful at rest. However the majority of these pressure deviations from baseline were in fact *reductions* of blood pressure, and this alone might over a longer time period account for a largely unchanged average pressure over 24 h in BDR.

During the standardized periods of stress neither BDR nor NCR showed any marked blood pressure responses and BDR were in this respect not hyperreactive, contrary to the findings in dogs by Cowley *et al.* (1973). This difference between species might be due to a weaker stimulus being applied in the present study which however seems unlikely since the standardized stress stimuli used induce pronounced blood pressure increases in the spontaneously hypertensive rat (SHR) (Hallbäck and Folkow 1974). If anything, the tachycardia response to stress was less in BDR than that in NCR, presumably related to the higher prestimulus heart rates in BDR as a result of baroreceptor denervation.

Thus, according to the present findings baroreceptor denervated rats do not respond differently than do normotensive control rats during stress stimuli. In this respect our results would, at first sight, seem to be at variance with those of Cowley *et al.* (1973) in dogs. The environmental stimuli used in our study were, of course, unpleasant in nature while those applied to the baroreceptor denervated dogs rather might have evoked pleasurable responses being induced when the animal keeper entered the experimental room. Hence the two types of stimuli would be entirely different in nature and are likely to induce different patterns of autonomic responses. When investigated during 24 h the neurogenically "hypertensive"

meters, *i.e* resistance at maximal vasodilatation, the slope of the resistance curve and t maximal pressor response, the resistance vessels of the BDR did not differ structural from those of their normotensive controls Structural adaptation of the left ventricle seem not to have occurred either since left ventricular weight in percentage of body weight d not differ between BDR and NCR being 0.177 ± 0 004 per cent *vs* 0 174 ± 0.004 respectiv ($p < 0.5$) (see Table I)

Discussion

The results of the present study largely support the contention of Cowley *et al.* (1973) th the term "neurogenic hypertension" may be a misnomer for animals deprived of their bar receptor reflexes

However a complete baroreceptor denervation is difficult to achieve and Krieger wl introduced the technique for baroreceptor denervation in the rat, found only a 70 per ce success rate in achieving hypertension" after one year Furthermore a fairly high mortali was reported (Krieger 1964) Thant *et al* (1969) have suggested that this was due to t acute chemoreceptor denervation in the anesthetized animal and were able to decrease t mortality by not anesthetizing the animal during the operation. In the present study animals used for test operations developed respiratory difficulty and died at the mome when all the nerves coursing near the carotid sinus were divided in order to clean the sin for local phenol administration. If only these nerves were first displaced and not section the animals survived the operation well Thus, by a slight adjustment of Krieger's techniq we were able to obtain 100 per cent survival and as much as 75 per cent of the operated ra had blood pressures greater than or equal to 120 mmHg.

The mean blood pressure of the 15 rats used was some 15 per cent above that of the NC during the period of measurement This increase in mean blood pressure may therefore taken as a fairly good indication of successful baroreceptor denervation. Furthermore, operated rats had increased heart rates when compared to their appropriate NCR and 15 used for stress comparisons showed greater and more frequent fluctuations of blo pressure when compared to NCR (see Fig. 1) Finally angiotensin injected intraarteria caused an abrupt increase in blood pressure in both NCR and BDR but reflex slowing heart rate occurred only in NCR, a response that is thought to be baroreceptor mediat (*cf* Korner *et al* 1974) Taken together these observations indicate that the 15 operat rats used could be considered to be adequately baroreceptor denervated for the purposes the present study

The present results further show that in rats who have had their systemic arterial bar receptors denervated up to 3 1/2 months previously no structural adaptive changes occ in either the resistance vessels or in the left ventricle. This is despite the fact that these ra had on the two occasions when pressure was measured approximately a 15 per cent increa in mean arterial blood pressure compared with matched paired normotensive control ra Structural cardiovascular changes have repeatedly been found in chronic hypertension, e in human essential hypertension in spontaneously hypertensive rats and in secondary ren hypertension, and to an extent that is largely proportional to the increase in mean arter blood pressure (*cf* Folkow *et al* 1973) Furthermore, a recent study on the Mil

...is study was supported by grants from the Swedish Medical Research Council (No 14X-00016), the ...edish National Association against Heart and Chest Diseases and the British Medical Research Council ... V. Jones was British MRC Travelling Research Fellow for 1975-76)
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dogs as a group displayed a diurnal variation in blood pressure with variations as great as 15 mmHg. The peaks in pressure did, however not appear to be associated with the surrounding noise level, as would be expected if "noxious" stimuli are particularly apt to cause greater pressure responses in baroreceptor denervated animals. Attempts were made in the present group of rats to follow mean arterial pressure during 24 h but for technical reasons they were unsuccessful due to the small size of the animal which makes it particularly difficult to leave them really undisturbed. In the baroreceptor denervated dog the lowest blood pressures were recorded in the early hours of the morning when the animals presumably were asleep (Cowley *et al* 1973)

Although Ferrario *et al* (1969) have failed to observe that blood pressure falls during sleep in baroreceptor denervated animals and they indeed noted an increase in blood pressure due to an increase in peripheral resistance, there is other evidence that mean arterial pressure falls markedly during deep sleep in such animals (Guazzi and Zanchetti 1965). It has then been suggested that a precipitous fall may only be prevented by activation of the peripheral arterial chemoreceptors (Zanchetti *et al* 1967) It is possible, therefore, that the BDR may have been relatively hypotensive when deeply asleep even though no difference in mean blood pressure was obtained after nembutal anesthesia. Although our animals were "hypertensive" during the time they were exposed to acute study the lack of adaptive structural changes in heart and resistance vessels strongly suggests that there were compensatory periods of hypotension keeping the averaged mean arterial blood pressure over 24 h in a normotensive range.

The observation that BDR did not gain weight as rapidly as NCR is of additional interest. They were fed the same diet and housed side by side under identical conditions as their NCR counterparts at least for the two months prior to the terminal experiment. They seemed to thrive as well as the NCR and showed no evidence of discomfort or poor health. The presence of intimate connections between the buffer nerves and the medial reticular formation implies that changes in baroreceptor activity may induce behavioural changes. It has thus been shown that powerful baroreceptor excitation inhibits sham range (Bartorelli *et al.* 1960) and may even induce signs of sleep (Bonvallet Dell and Hiebel 1954) Therefore, chronic deactivation of the buffer nerves may perhaps induce an increased alertness or a restless behaviour in such a way that *e.g* the balance between food intake and general metabolism is altered in BDR This aspect of baroreceptor control merits, however further study

In conclusion we have shown, in agreement with Cowley *et al* (1973) that the "hypertension" consequent upon baroreceptor denervation seems not to be of a stable and persistent type in the sense that mean arterial pressure over a longer time period really is elevated Had this been the case structural changes in the cardiovascular system would have developed within the time between operation and hemodynamic analysis as indicated by several other studies on rats with various types of mild or severe chronic hypertension BDR do exhibit wider and more frequent oscillations in blood pressure when compared to NCR but the peaks, at any rate, do not necessarily seem to be evoked by overt alerting stimuli and transient pressure reductions are even more frequent than the pressure elevations. The arterial baroreceptors do indeed, seem to function as "buffer nerves" but seem to have little play in setting of average level of mean arterial pressure throughout the whole d

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Ultrastructure of synaptosomes from fetal rat brain

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Abstract

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The crude mitochondrial fraction P and subfractions of P were prepared from the brain stem, hemispheres and whole brain of 19-day-old fetal rats. Samples were fixed in glutaraldehyde–osmium, Na or by Tranzer's triple fixation method (aldehyde–chromate–dichromate–osmium) and examined electron microscopy The C-fraction from whole brain was the main synaptosome fraction, containing 3.2% presynaptic terminals as counted from all membrane bound particles. The brain stem showed presynaptic terminals than the hemisphere (2.8% versus 0.9) suggesting caudal–rostral maturation gradient for synaptogenesis. The maturity of the nerve endings obtained was very variable in contrast the rather uniform synaptosomes derived from adult tissue. They varied from profiles without any structures to mature synaptosomes displaying asymmetric synaptic junctions. Monoamine synaptosomes containing small granular vesicles were not detected in the present study suggesting immaturity of granular monoamine pool at this stage of development.

Key words: Synaptosomes, development, ultrastructure, rat, brain

The application of cell fractionation techniques in studies of nervous tissue (see Whittaker 1965 1972, Jones 1975) has resulted in significant progress in the knowledge concerning neuronal function. It is assumed that on homogenization the terminal region of the nerve is "pinched off" and seals up to form a discrete subcellular particle, which carries with it a length of the characteristically thickened postsynaptic membrane Comprehensive reviews of the morphological and biochemical properties of pinched off nerve endings (synaptosomes) have been published (Whittaker 1955 1972, Jones 1972, 1975)

Isolated nerve endings have also been used with success in studies on several aspects of the ontogenesis of the nervous system (Jones 1972, Tissari 1973 1975). The number of nerve endings *in vivo* is small during early maturation (Tennyson 1970 a, Hervonen *et al* 1974, Kanerva *et al* 1974 a, b). Thus, the subcellular fractionation technique is especially suited in studying the fine structure of the developing nerve endings, since large numbers can be obtained for examination with electron microscopy

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With the exception of short notes on the fine structure of synaptosomes from fetal rat [brains] (Abdel-Latif and Abood 1964, Abdel-Latif *et al.* 1967) there is virtually nothing in [th]e literature concerning development of synaptosomes at fetal stages in vertebrates. Further[m]ore, the results of Abdel-Latif and coworkers are in conflict with more recent observations. [A]bdel-Latif and Abood (1964) and Abdel-Latif *et al.* (1967) reported that maximal struc[tur]al changes occurred between the 16th fetal and the first postnatal day at which stage the [st]ructure did not differ from that found in the 25-day-old and adult rat. Other workers [(]Jones and Revell 1970, Pfrsa *et al.* 1970, Gonatas *et al.* 1971) found that synaptosomes [is]olated during the first few postnatal days were very primitive and that maximal struc[tur]al changes occurred considerably later.

We have recently confirmed and extended the results of Jones and Revell (1970) and [G]onatas *et al.* (1971) in the 1-day-old rat (Hervonen *et al.* 1974, Kanerva *et al.* 1976, 1977). [T]he purpose of this further study of the ontogenesis of synaptosomes was partly to analyze [t]he fine structure of fetal rat synaptosomes. This is of importance in understanding the [ge]neral events during synaptogenesis and in promoting a better basis for evaluating bio[c]hemical and pharmacological data in this age groups (Tissari 1973, 1975, Tissari and Rannu [1]975). We also wanted to compare synaptosomes (synaptogenesis) from the brain stem and [t]he hemispheres, since a caudal–rostral maturation gradient has been found for the brain [c]ontent of several neurotransmitter constituents (McCaman and Aprison 1964, Porcher [a]nd Heller 1972, Coyle and Axelrod 1971, Tissari 1973). Finally we also made an attempt [to] demonstrate the granular pool of monoamines in the nerve endings at this early stage of development.

The results have been partially communicated in conference reports (Kanerva *et al.* 1975 b, c).

Material and Methods

Preparation of subfractions

19-day-old Sprague-Dawley rat fetuses were used in this study. Subfractions were prepared from the brain essentially as described by Rodriguez de Lores Arnaiz and DeRobertis (1964), modified according to Tissari *et al.* (1969).

Brain tissue (whole brains, hemispheres, or brain stems) was homogenized in 9 volumes of 0.32 M sucrose containing 10^{-4} M $CaCl_2$ using [a] glass homogenizer and Teflon pestle with approximately 0.25 mm clearance and 70 strokes. The homogenate was centrifuged at 1 000 *g* for 10 min. The supernatant was removed and the pellet resuspended with 10 ml 0.32 M sucrose containing 10^{-4} M $CaCl_2$. The pellet suspension was centrifuged at 1 000 *g* for 10 min. The two supernatants were combined and centrifuged at 10 000 *g* for 20 min. The supernatant was then decanted and the pellet ([P] or crude mitochondrial fraction) was resuspended in [a] suitable volume of 0.32 M sucrose [wi]th 10^{-4} M $CaCl_2$ (usually 5–10 ml) for layering on discontinuous sucrose gradient.

The crude mitochondrial fraction derived from 2–3 g of original tissue per gradient was resuspended in 0.32 M sucrose and layered over [a] discontinuous sucrose gradient consisting of layers of 1.4, 1.[2], 1.0 and 0.8 M sucrose. The gradient was centrifuged at 53 000 *g* for 120 min in [a] Spinco Model L ultracentrifuge [e]quipped [wi]th [a] SW 25.1 rotor. The fractions of sucrose gradient were removed by aspiration and diluted [with] enough [wa]ter to produce [a] sucrose concentration of 0.32 M for subfractions A and B and 0.55 M for C, D and E. After standing for 20 min the suspensions were centrifuged for 30 min at 15 000 *g* except for subfractions A and B [wh]ich were centrifuged at 40 000 *g*. Pellets were suspended in 14 ml of Krebs-bicarbonate solution or other incubation media, [e.g.] Na-free Krebs solution containing all other electro[ly]tes and with its isotonicity maintained by sucrose. Incubation suspensions usually contained 0.2–0.3 mg [protein/m]l. Aliquots were removed for electron microscopy (see below).

Fixation for electro microscopy

The samples were fixed either as a suspension (glutaraldehyde, permanganate) or pellet (all fra following fixatives were used (all concentrations final) (1) 3 % glutaraldehyde in 0 1 M phosph pH 7 4 for 120 min, (2) 6–9 % sodium permanganate in phosphate buffer pH 7 4 for 45 min (1 1966, Hökfelt *et al* 1970) or (3) aldehyde-chromate-dichromate–osmium (triple fixation) and co-workers (Tranzer and Snipes 1968, Tranzer 1972, Tranzer and Richards 1976). The sy were fixed as pellets in mixture of glutaraldehyde (1 %) and formaldehyde (0 4 .) buffered to p 0 1 M chromate-dichromate, fo 30 min. The fixation was continued overnight in a 0.2 M dichromate solution at pH 6 0 The glutaraldehyde and aldehyde–chromate-dichromate fixed sa postfixed in phosphate buffered 1 % OsO at pH 7 4 for 1 2 h. Then the specimens were deh embedded in Epon Araldite. The $NaMnO_4$-fixed sections were viewed and photographed unst the other specimens were grid-stained with lead citrate and uranyl acetate. A Zeiss EM 9A elect scope operated at 60 kV was used.

Incubation f synaptosome with subst ates

Samples for incubation with substrates were transferred to a Dubnoff water bath shaker and at 37°C and 95 % O_2 + 5 % CO_2 for 8 min before addition of substrate. After adding substrate was conti ued and aliquots were removed after 15 or 30 min. Aliquots (2 ml, except A-lay 3.5 ml) were removed to pre-cooled plastic tubes in an ice bath. After 20 min, samples ere cen 10 000 *g* for 15 mi and the pellet was fixed for EM Substrates used were 5-hydroxy-dopamine or L-α-methyl-noradrenaline (α-methyl-NA) The substrate was used in a concentration of 100 added in 0.2 mg/ml ascorbic acid.

Quantitati e electron microscopic calc lations

Fo quantitative calculations, 5–10 non-overlapping serial electron micrographs were taken d pellets t insure their representative nature. A primary magnification of approximately 13 000 × 16 500) was used. The negatives of these samples were then photographically enlarged (3) synaptic vesicles were readily recognized. Measurements of synaptic vesicles were made using a under magnifying glass. Two measurements were made on each esicle its maximum outside and a measurement at right angles to the long axis. The mean was considered the size of the ve

To calculate the number of membrane-bound particles (MBP') with or without synaptic vesic larger than 0.3 μm in diameter with 3 more vesicles 200–600 Å in diameter were characteri synaptic endings.

Results

The best preservation was obtained with glutaraldehyde fixation in susp nsion by osmication The description below mainly refers to the fine structure aft r this procedure The difference in fine structure after different fixatives, the regional v and the effect of incubations are described subsequently

Fine Structure of the Fractions

Fraction P2

The main components were membrane bounded particles (MBP), free mitochon membrane fragments (devoid of associated cytoplasm) (Fig. 1–4) Some of the contained synaptic vesicles and some small intra-axonal mitochondria They ap represented developing or in a few cases mature pinched off nerve endings (synapt Sometimes these synaptosomes were attached to a postsynaptic membrane (Fig. MBP's (with or without synaptic vesicles) also contained amorphous ground mater of smooth or rough surfaced endoplasmic reticulum, multivesicular bodies, neu coated vesicles and bundles of fibrillar material. The free mit a wer



Fig. 1. P2-fraction, hemisphere, glutaraldehyde-fixation in pellet, no incubation. Several membrane-bound particles (MBP) are seen. Some of them contain agranular synaptic vesicles (arrow) and can be considere pinched off nerve endings (S), while others (P) are devoid of synaptic vesicles. MBP' have an amorphou substructure and also contain small mitochondria (m), tubules (thick arrow) and fibrils (double-arrow Other components are the free mitochondria (M) and microsomes (MIC) × 30 000.

rupted, but were recognized because of their cristae and double membranes. Sometimes th were shrunken, with a dark appearance.

Compared to the adult (Hervonen *et al.* 1974) the main differences were the absence myelin, the small number of synaptosomes containing synaptic vesicles, and the large num ber devoid of synaptic vesicles. On the other hand, in the 1-day-old rat some myelin w found and the number of synaptosomes was greater (Hervonen *et al.* 1974).

Fraction A

This fraction consists mainly of unit membrane fragments (Fig. 5). They are usually curv and of very variable length. No myelin fragments were found. If myelination has begun this stage, the myelin breaks down into single unit membranes during homogenization. T MBP's in this fraction are fewer and smaller than in the following subfractions. This

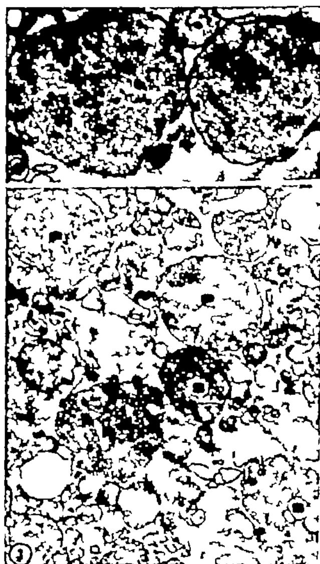


Fig. 2. P_2-fraction, brain stem, glutaraldehyde-fixation in suspensio no incubation. Two MBP's of one (P) is devoid of synaptic vesicles, and the other f irly mat re synaptosome The pre and postsyn membrane thickenings and some cleft material between them can be see Note the small size of dense-cored vesicles (bout 500 Å, arrow), and the larger size of third (open arrow) 37 900.

Fig 3 P fraction, brain stem, triple-fixation, 5-OHDA incubation The fine structure after triple-fix resembles that fter glutaraldehyde-OsO_4-f xation (Fig. 1 2). Two (S) of the MBP' contain several sy vesicles, while others are devoid of them. The synaptic vesicles re small nd granular A coated is also observed (rrow), but not small granular vesicles. Several membrane particles can be seen (rrow). 21 400

applies to MBP's with synaptic vesicles, *i.e* synaptosomes (see Table I) The synaptoso cannot be distinguished morphologically in the different subfractions.

Fraction B

The number of membrane fragments is smaller than in fraction A whereas the number size of MBP s is larger The MBP's usually contain only few synaptic vesicles (develop synaptosomes) but a few mature looking nerve endings can be found (Fig 6) Th

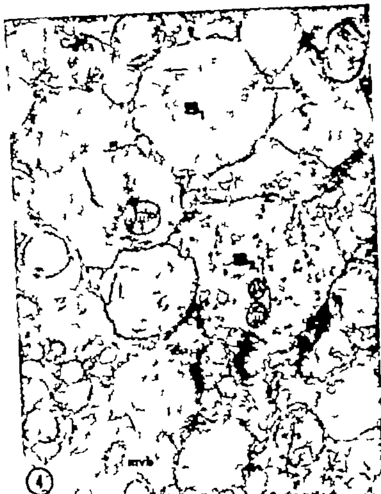


Fig. 4. P_2-fraction, hemisphere, permanganate fixation in pellet, α-methyl-NA incubation. Most of the profiles are devoid of synaptic vesicles or contain only a few of them (S_1, arrow). One mature looking synaptosome (S_2) with small agranular vesicles, some large granular vesicles (tailed arrow) and small intrasynaptosomal mitochondria (m) are seen. One MBP contains a multivesicular body (mvb). × 34 700

nerve endings are packed with synaptic vesicles and display postsynaptic membrane thickening. The percentage of presynaptic terminals was calculated to be 1.8% (see Table I).

Fraction C

This fraction together with fraction B, represents the main synaptosome fraction in the fetal rat brain (see Table I). MBP's predominate in the fraction, which also contains some free mitochondria (Fig. 7). The MBP's are larger than in fraction B. They contain amorphous ground material and few organelles. They contain vesicles of the following types.

(1) *Small agranular vesicles (SAV).* These are most common. They are spherical and between 200 and 600 Å in diameter (mean 420 Å, calculated from 500 vesicles). Usually there

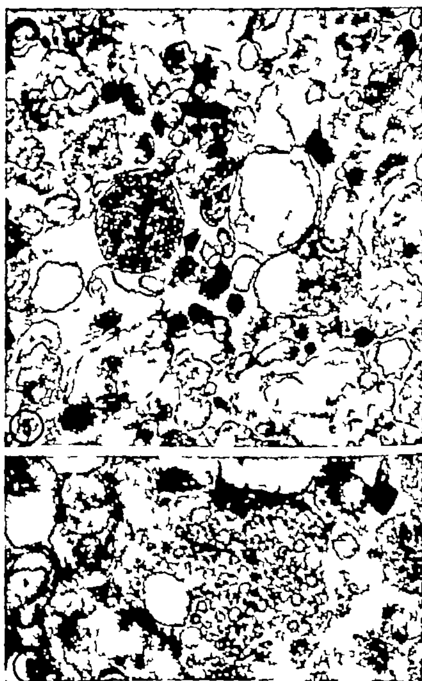


Fig. 5 A fraction, whole brain, triple fixation, 5-OHDA ncubation. One synaptosome (arrow) with SAV most of the fraction consists of membranes. 37 200

Fig. 6. B-fraction, whole brain, triple fixation, 5-OHDA ncubation This synaptosome ha t teristics of a mature nerve ending: It is packed with synaptic esicles and display symmetrica postsynaptic membrane thickenings. Some vacuoles are seen in the synaptosome. 58 300.

are relatively few such vesicles per nerve ending, but the more mature nerve en packed with these vesicles as in adult synaptosomes.

(2) *Dense cored vesicles (DCV)* These are usually intermingled with SAV in sm bers. Most are between 500 and 800 Å in diameter (mean 620 Å calculated from 100

BLE I Rat synaptosome fractions in 19-day-old fetus.

ain region	Fraction	Total number of counted membrane-bound particles (MBP's)	MBP' without synaptic vesicles	MBP' with synaptic vesicles = pre-synaptic terminals	Percentage of pre-synaptic terminals
emisphere	F2	1 000	991	9	0.9
rain stem	F2	1 000	977	23	2.3
hole brain	A	1 000	993	7	0.7
	B	1 000	982	18	1.8
	C	1 000	968	32	3.2
	D	1 000	994	6	0.6
	E	1 000	995	5	0.5

nd are thus smaller than the large granular vesicles (LGV) in adult nerve endings. They are lso found alone in MBP's without other synaptic vesicles and may have functions different rom neural transmission. Permanganate fixation does not demonstrate a dense core in hese vesicles in the 19-day-old fetal brain.

(3) *Coated vesicles.* These are found both in MBP's without other organelles and in nerve ndings containing the above types of synaptic vesicles. They are mostly 600–900 Å in diameter (mean 760 Å calculated from 100 vesicles).

(4) *Large agranular vesicles.* These vesicles, 600–2 000 Å in diameter are found among other synaptic vesicles or occasionally alone. They might be part of the smooth endoplasmic reticulum and/or represent developing or degenerating synaptic vesicles (Kanerva *et al.* 1975).

Synaptosomes packed with so-called small granular vesicles (SGV) typical of monoaminergic nerve endings (Hokfelt *et al.* 1970 Kanerva *et al.* 1976, 1977) have not been observed in the fetal rat.

The attached postsynaptic membrane which is frequently seen in adult synaptosomes (Hervonen *et al.* 1974) was observed, but not frequently. The adjacent synaptic membranes were either symmetrically thickened or usually the postsynaptic membrane was thicker. This is indicative of the mature appearance of synapses. The synaptic cleft measured 200 Å and contained some cleft material.

Fraction D

This fraction consists mainly of MBP's and free mitochondria. The MBP's are larger than in fraction C. They only infrequently contain synaptic vesicles thus the number of synaptosomes or developing synaptosomes is small compared to fraction C. The large MBP's contain more organelles than MBP's in the lighter subfractions (such as mitochondria, rough or smooth endoplasmic reticulum, free ribosomes, fibril bundles, microtubules, vacuoles, granular and agranular vesicles). They might be derivatives of dendrites, perikarya or glial tissue rather than pinched off axons. The free mitochondria are large and often disrupted.

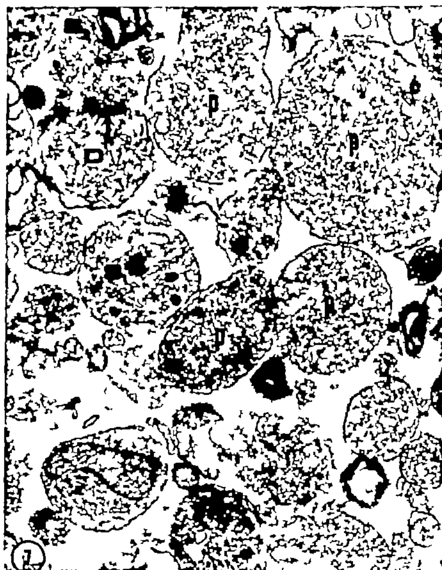


Fig. 7 C-fraction, whole brain, glutaraldehyde-fixatio l suspension, no incubation. Several MBP's r seen. One (S_1) contains 3 dense-cored vesicles (DCV) (arrow), while another (S_2) with synaptic vesicles both SAV and one DCV This synaptosome might be connected at membrane thickenings (thick arr to a dendrite (D) containing microf brils, microt bules nd some ribosomes. The other MBP's (p) conta mal ly amorphous ground material. A coated vesicle is seen in one of them (thick open rrow). 37 0

Fraction E

The main constituents are large free mitochondria. MBP's of the same appearance as Fraction D are encountered and a few synaptosomes can be found.

Fine Structure after Different Fixatives

Compared to glutaraldehyde followed by osmication (Fig. 1–2, 7) the triple fixation metho gave somewhat inferior preservation with more vacuoles in the MBP's (Fig. 3 5–6) structures were observed with this fixation. After permangan (Fig. 4

ficult to obtain good ultrathin sections. In the fetal rat synaptosomes, we were not able demonstrate small granular vesicles (SGV's) characteristic of monoaminergic synaptosomes even after incubations with "false" transmitters (see below).

Regional Variations

The synaptosomes from the different brain areas, *i.e.* the brain stem, the hemispheres or the whole brain were identical in morphology. We counted the number of MBP's from the P_2 layer of the brain stem and the hemispheres (Table I). 2.8% of MBP's contained synaptic vesicles in the brain stem preparation vs 0.9% in the hemisphere preparations. This seems to confirm our previous assumption that nerve endings in the brain stem develop earlier than those in the hemispheres (Hervonen *et al.* 1974).

Effect of Incubations

Contrary to our previous results in the 1-day-old rat (Kanerva *et al.* 1976, 1977) small DCV's could not be demonstrated after incubation in either 5-OHDA or α-methyl-NA. This reflects the immaturity of the monoamine-storing vesicle pool at this stage of development. The appearance after incubation was largely unchanged irrespective of fixation method.

Discussion

The formation of synaptosomes is believed to result principally from differences in mechanical and physical properties between the nerve ending and the structures which surround it (Whittaker 1965). It seems generally accepted that by using the subfractionation technique for nervous tissue, the synaptosomal fractions obtained are relatively pure in pinched off nerve endings (Whittaker 1965, 1972, Jones 1972, 1975). Other components in the adult synaptosomal fractions (fraction C with the method used in the present study) are few free mitochondria, some membrane fragments and few unidentified bodies. Lemkey-Johnston and Dekirmenjian (1970), on the other hand, were of the opinion that synaptosome preparations are more heterogenous, also containing large numbers of axonal segments and membranes of undetermined origin. However, their subfractionation technique has been criticized (Jones 1972). During ontogenesis, the neurons (and the glial cells) might have different mechanical and physical properties. During development, neurons differ from those of the adult (Tennyson 1970 a, b). Prominent structures during development are the growth cones (Tennyson 1970 b). These are found both at the tips of the dendrites and the axons (Kawana *et al.* 1971, Vaughn *et al.* 1974). Several electron microscopic studies on the growth cone have appeared during the last years (Bodian 1966, Bodian *et al.* 1968, Del Cerro and Snider 1968, Tennyson 1970 b, Kawana *et al.* 1971, Vaughn *et al.* 1974). The growth cones consist of a bulging cytoplasm from which extremely fine filopodia protrude. The main structural characteristics of the cone-shaped area are the smooth endoplasmic reticulum complexes. These are organized as tubules, sacs and vesicles. Other components are microtubules, filaments, mitochondria, coated invaginations of plasma membranes, coated vesicles, lysosomes and synaptic vesicles. The similar arrangement of endoplasmic reticulum in the

growth cones and in the synaptosomes allows us to suggest that some of the memb bounded particles observed during ontogenesis are derived from growth cones. In the way that pinched off nerve endings are formed in adult animals, pinched off growth c might be formed during subfractionation of immature nervous tissue. Since growing drites also display growth cones (Kawana *et al* 1971 Vaughn *et al* 1974) it might be t larger proportion of membrane bound particles are derived from dendrites during genesis than in adults. Kawana *et al* (1971) were unable to differentiate with certainty axonal and dendritic growth cones *in vivo* Thus, probably they cannot be different after the subfractionation procedure In summary it seems that after subfractionati immature nervous tissue, the synaptosomal fractions contain pinched off growth cones axons and dendrites and perhaps from glial cells, in addition to the normal constitues the synaptosomal fractions (synaptosomes, mitochondria, some membrane fragments few unidentified bodies)

During the last decade several investigations have dealt with the fine structure of syn genesis in several animal species (Tennyson 1970 a). In the rat postnatal synaptogenesi been investigated in many brain areas including the neocortex, the lateral geniculate an the olfactory bulb, the parietal cortex, the cerebellar cortex, the hippocampus, the sup colliculus, the dentate gyrus, the corpus striatum and the lateral vestibular nucleu Tennyson 1970 a, Hervonen *et al* 1974). Some reports on fetal synaptogenesis have appeared (Kobayashi *et al* 1968 Daikoku *et al.* 1971 Del Cerro and Snider 1972, May Biscoe 1973 Vaughn and Grieshaber 1973 König *et al* 1975). Judging from the curr available literature, it seems reasonable to assume that while mature synapses can be f during prenatal development in the rat the period of rapid increase in number of syn in most parts of the brain is during the first three postnatal weeks (*e.g.*, Aghajanian Bloom 1967 Woodward *et al* 1971). This interval between the time at which synapse first formed and the time of rapid increase in number also applies to mammals with a mature nervous system at birth. For example in the cat visual system in which a few de synapses are present as early as three weeks before birth the rapid increase of syn occurs 8–37 days after birth (Cragg 1974) The present finding of developing synaptos during the prenatal development fits well with the above concept and holds promis future work on the immature synapse using the subfractionation technique.

The synaptic vesicles are mainly small agranular vesicles (SAV) and large granular ve (LGV) In the fetal synaptosomes, SAV were of the same size (mean 420 Å) as reporte adults (Whittaker 1972). This is in agreement with a study on the median eminence of th where SAV did not appear to change in size during pre or postnatal development (K yashi *et al* 1968 Daikoku *et al* 1971) LGV on the other hand, were reported to inc in size most being 600-800 Å in diameter in the 18-day-old fetus, later increasing to 1 000 Å at which size they remained during postnatal development. In the present p the size of LGV (mean 620 Å) is smaller than reported for adults (Whittaker 197?). T LGV were solitary among large numbers of SAV in the synaptosomes and can hardly been so-called small granular vesicles (SGV) containing monoamines (Hökfelt 1973) I ninger *et al* (1969) have tentatively proposed that there is a close relationship dense-cored vesicles and dense projections. We have not been able to confirm th

in this or our other works on the ontogenesis of nerve endings (Hervonen *et al.* 1974 erva *et al.* 1974 a). Regarding the genesis of synaptic esicles, it seems that their origin almost every component of the neuron has been proposed mitochondria, microles, cell membrane, complex vesicles, large granular vesicles, the Golgi apparatus, and smooth endoplasmic reticulum (SR) (for ref see Hökfelt 1973 Kanerva *et al.* 1974 a). co-existence of synaptic vesicles and smooth endoplasmic reticulum in the membranend particles (MBP's) is of interest. These two elements also share an affinity for the zinc de-osmium (ZIO) stain (Kawana *et al.* 1971) and it is possible that the small agranular cles are SR derivatives. One step during this formation might be the large agranular cles (Kanerva *et al.* 1975 a). The dense core of the SGV on the other hand has been obved to form within SR tubules (Hervonen and Kanerva 1973 Hökfelt 1973 Kanerva *al.* 1976).

The best results in demonstrating monoamines with electron microscopy have been obned by (1) permanganate fixation (Richardson 1966, Hökfelt 1973), (2) glutaraldehyde ation with post-osmication after loading the nerve endings with "false transmitters" such 5-OHDA, α-methyl-NA or 6-OHDA (Tranzer 1972, 1973 Hokfelt 1973) or (3) a modified ethod based on the chromaffin reaction (Wood and Barrnett 1964) introduced by Tranzer d co-workers (Tranzer and Snipes 1968, Tranzer 1972, 1973 Tranzer and Richards 1976). is claimed that this triple-fixation method (aldehydes, chromates and osmium tetroxide) Tranzer 1972, 1973) permits the demonstration of both the so-called granular pool and the on-granular pool of monoamines. In the present paper we have used the above fixation rocedures but were not able to demonstrate monoamine-structures in fetal rat synaptosomes. has been suggested that the tubular reticulum which is believed to store the non-granular ool of monoamines (Eränkö 1972, Tranzer 1972, 1973) is labile and the homogenization rocedure could thus liberate their amine content during the processing of synaptosomes. We have previously demonstrated small granular vesicles in the 1-day-old rat although their umber was small (Kanerva *et al.* 1976, 1977). Varicose nerve terminals ha e been demonstrated in the fetal rat brain by the formaldehyde-induced fluorescence (FIF) technique Seiger and Olson 1973). Since SGV were not found in the present study and monoamines can be demonstrated in the nerve endings with the FIF method in the fetal rat, we conclude that the granular pool of catecholamines is poorly developed or less stable than in adults and/or the cytochemical techniques used are defective in demonstrating the SGV in the central nervous system during this early stage of development.

This ork as finished hile one of us (L. K.) was on Public Health Service International Research Fellowship (Number 1 F05 TW02053-01) at the University of California, School of Medicine, Department of Human Anatomy Davis, California 95616, U.S.A. (Permanent address: Department of Anatomy University of Helsinki, Finland.)

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Effect of chronic hypoxia on hepatic triacylglycerol concentration and mitochondrial fatty acid oxidizing capacity in liver and heart

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Abstract

KINNULA, V L. and I HASSINEN *Effect of chronic hypoxia on hepatic triacylglycerol concentration and mitochondrial fatty acid oxidizing capacity in liver and heart* Acta physiol scand 1978 *102* 64–73

The effect of moderate hypoxia (50 5 kPa air) and severe hypoxia (40.8 kPa ai) *in vivo* on liver and h triglyceride concentration and mitochondrial respiration rates was studied Liver triglyceride concent tions increased i severe hypoxia from 7 3 µmol/g wet weight t 23.3 µmol/g wet weight over 7 days. A the period of seven days in severe hypoxia the palmitate, octanoat a d palmitoylcarnitine oxidation r of mitochondrial suspensions were significantly reduced when the citric acid cycle was operative. decrease in the fatty acid f tty acyl-CoA o carnitine derivative oxidation was observed when only β oxidation system was studied. M toch ndria isolated f om the heart r liver after seven days in se hypoxia showed reduced respiratory control ratios, the decrease being from the normal 4.9 to 1 9 in liver mitocho dria usi g succinat as substrate. The reduction respirat ry control was mainly due lowered Stat 3 respiration rates. Some reduction n the ratio was lso observed in the fasting contr from 5 8 to 3 4 with succinate. The respiratory control ratio could be partially normalized by the addit of albumin t the isolation medium for the l ver mitochondria after severe hypoxia. Under these conditi however the State 4 respiration f the m tochondria from the hypoxic animals was higher than that for controls.

Key words. Heart, liver hypoxia, triglycerides, f tty acid oxidation

The role of oxygen in the induction of the biogenesis of mitochondria and the synthesis cytochromes in the lower eucaryotes is well established (Biggs and Linnane 1963) The vi also emerges that oxygen availability may also be an important regulator of the synthesis some of the components of mitochondria in mammals, both in cell cultures (Prous 1970 and *in vivo* (Kinnula 1976) There is also evidence that the postnatal increase in the amount of mitochondria may be accounted for by the increase in oxygen concentration (... 1971 Kinnula and Hassinen 1977).

There is some evidence that the capacity of the mitochondrial fatty acid oxidizing system is a positive correlation with the cytochrome content of the mitochondria, at least in mitochondria from hyperthyroid animals (Kadenbach 1966, Dumont *et al* 1972). The present study was therefore undertaken to test the possible inducing effects of oxygen on a typical mitochondrial aerobic pathway the β oxidation of fatty acids.

This demonstrated that the capacity for fatty acid oxidation is impaired in hypoxia. This is probably due to decrease not in the activity of the fatty acid oxidizing enzymes but in that of the mitochondrial respiratory chain. An accumulation of triglycerides occurs in the liver during hypoxia, probably due to the decreased fatty acid oxidation.

Material and Methods

Reagents Standard reagents were obtained mainly from E. Merck AG, Darmstadt, Germany. Fatty acids, fatty acid derivatives, albumin and ADP were purchased from Sigma Chemical C St Louis, M U.S.A., and carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (Cl-CCP) from Boehringer GmbH, Mannheim, Germany. Bacillus subtilis alkaline was obtained from Novo Industri A/S, Bagsværd, Denmark.

Animals and hypoxic experiments Adult Wistar rats of both sexes (age 3–4 months) were used. The hypoxic periods consisted of one day or seven days in a hypobaric pressure chamber at atmospheric pressure of 50.5 kPa or 40.8 kPa. In the one-day expts only water was available for the rats, but during the longer hypoxic periods they had access to food (standard rodent chow obtained from Hankkija Ltd, Helsinki, Finland) and water. Since rats eat less when exposed to hypoxia, the effect of food restriction was tested on one group of control animals by limiting food intake. Rats on the restricted diet were given food for 1 h day (Ziegler 1967), during which time they consumed 5–10 g, compared with normal consumption of 20–25 g day. With this arrangement the weight loss in the fasting controls was the same as in the rats undergoing severe hypoxia.

Methods Liver mitochondria were isolated by conventional Schneider procedure, the isolation medium consisting of 0.25 M sucrose, 1 mM EDTA and 5 mM Tris-chloride (pH 7.4). The mitochondrial fraction was washed twice. In some of the expts albumin was added to the isolation medium to a final concentration of 3 (Martin and Denton 1971). In this case one washing was included in the isolation. Heart mitochondria were isolated as previously described (Kinnula 1976), essentially according to Tyler and Gonze 1967). The isolation medium consisted of 0.225 M mannitol, 0.075 M sucrose and 0.05 mM EDTA (pH 7.4). Usually Bacillus subtilis alkaline (EC 3.4.4.16.) was used during the isolation, though it was omitted, as indicated, in some experiments. Heart and liver triglycerides were determined by the method of Carlson (1963). The rate of oxidation of fatty acids was determined polarographically (Yellow Springs oxygen electrode 5331), essentially according to Garland (1968). Initially the mitochondrial suspension was added to an air-saturated medium consisting of 80 mM KCl, 20 mM Tris-chloride, pH 7.4, 5 mM malonate, 5 mM malate, 3 mM phosphate, 1 mM EDTA and 2 mM ADP. Malonate was omitted from the oxidation expts on the heart mitochondria, and malonate or malate from those on the liver mitochondria in some cases. The concentration of palmitoylcarnitine was 37 μM (Garland 1968). The concentration of palmitate and octanoate, when present, was 0.7 mM (Pande 1971, Hassinen and Kähönen 1974). The palmitoyl-CoA concentration was 3 μM and carnitine concentration 1.5 mM (Pande 1971). In expts on succinate, 2-oxoglutarate or glutamate malate, the buffer consisted of 0.225 M sucrose, 10 mM phosphate (pH 7.4), 5 mM $MgCl_2$ and 20 mM KCl. Further additions were 5 mM substrate (State 4) and 0.25 mM ADP (State 3). All the oxidation expts were carried out at 22°C. Liver mitochondrial FAD and FMN concentrations were determined fluorometrically and non-fluorescent flavins spectrophotometrically according to King *et al* (1962). Protein determinations were carried out according to Lowry *et al* (1951). The Student's t test was used for the statistical analyses, and P values of < 0.05 were considered to indicate statistical significance.

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[1] In further expts (to be reported elsewhere) pair-feeding of the experimental animals was used. The food consumption data showed that the method of food restriction used in the present communication reproduces consumption of the hypoxic animals quite accurately.

Results and Discussion

An atmospheric pressure of 50.5 kPa represents moderate hypoxia to which adaptatio not difficult, as is observed in previous studies (Kinnula 1975 1976), whereas hypox the level of 40 8 kPa proved to be so severe that the animals were at the limits of their vival capacity and 9 5% died The mechanism of death was not investigated, but al deaths occurred during the first 3 days. At both pressures a clear hypertrophy of the h was seen, which was more prominent at 40 8 kPa, about 30%, when expressed as g/10 b wt Only in severe hypoxia did any decrease in liver occur about 20% over 7 days. corresponding weight decrease in the controls on the restricted diet was 22%, however

Subtilopeptidase A was used here for the isolation of heart mitochondria. Since evide has been presented of a susceptibility of ATP dependent long-chain fatty acid activ enzyme to the action of Subtilopeptidase A ("Nagarse") in liver and heart (Van Tol Hülsman 1970 Pande and Blanchaer 1970), its effect on the isolation of heart mitochon was tested, revealing no deleterious effects. Indeed, it had certain advantages, viz. an crease in the yield of mitochondria, which are also more stable, as evaluated from respiratory control ratios (Table I)

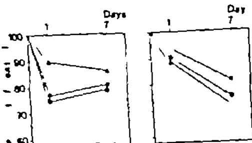
Uncoupling of mitochondria isolated from fat-containing tissues can be prevented including albumin in the isolation medium (Martin and Denton 1971), and thus, as an cumulation of triglycerides in the liver was observed in this study (see below), albumin also used in some of the expts. the final concentration in the isolation medium of l mitochondria being 2%.

Fatty acid oxidizing capacity From Fig. 1 and 2 it can be observed that under conditi allowing complete oxidation of fatty acids to CO and water the rate of oxidation in hyp declined systematically this effect being most prominent after seven days at 40.8 kPa. effect of albumin treatment on fatty acid oxidation under non-ketogenic conditions was tested The direction of the changes was the same for all the fatty acids studied, *i.e.* palmi

TABLE I Effect of B. subtilis alkalase on respiratory rates nd respiratory control ratios using succinat a substrate and the oxidation of palmitate, palmitat + carnitine nd palmitoylcarnitine by h mitochondria Values are means ± S.D from the umber of expts. indicated in parenth Oxygen consumption is expressed as μat ms/min/g protein (22°C). The mitochondrial suspe was dded to an al -saturated medi m consisting of 80 mM KCl, 20 mM Tris-chloride pH 5 mM malate, 5 mM phosphate, 1 mM EDTA and 2 mM ADP Palmitat was added at beginning of the expt. and carnitine min later Oxidation of palmitoylcarnitine w s measur separate expts.

	B subtilis alkalase	Alkalase omitted
Succinate, State 4	39 6 ± 5 8 (6)	31.8 ± 7._ (6)
Succinate State 3	134.2 ± 17.8 (6)	59 7 ± 9.8* (6)
Respiratory control ratio	3.5 ± 0 4 (6)	1.9 ± 0.2* (6)
Palmitate, State 3	47 1 ± 6 9 (5)	35.2 ± 7.3 (5)
Palmitate + carnitine, State 3	67 4 ± 10.2 (5)	54 0 ± 9 9 (5)
Palmitoylcarnitine, State 3	116 1 ± 12.4 (5)	106.0 ± 15.9 (5)

$p < 0.001$



1 Oxidation of palmitate, palmitoylcarnitine and octanoate by rat liver mitochondria after different hypoxic periods. Control values represent 100 %. ▲—▲ ..., ●—● palmitate and ■—■ palmitoylcarnitine. Left: moderate hypoxia (…5 kPa). Right: severe hypoxia (40.8 kPa). Conditions: See Table II, no albumin in the isolation medium

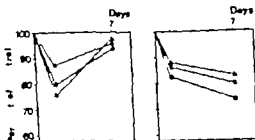


Fig. 2 Oxidation of palmitate, palmitoylcarnitine and octanoate by heart mitochondria after different hypoxic periods. Control values represent 100 %. ▲—▲ octanoate; ●—● palmitate and ■—■ palmitoylcarnitine. Left: moderate hypoxia (50.5 kPa). Right: severe hypoxia (40.8 kPa). Conditions: See Table II. Bacillus subtilis alkalase as used during the isolation.

palmitoylcarnitine and octanoate. The initial values, corresponding to the 100% values in Fig. 1 and 2, are presented in Table II.

The fatty acid β oxidation capacity during inhibition of the citric acid cycle is depicted in Table III. No significant differences can be observed between the control and hypoxic groups in the palmitoylcarnitine or palmitoyl-CoA oxidation experiments, and only a slight decrease can be noticed in the palmitate oxidation capacity in the hypoxic mitochondria. In these experiments albumin was added to the isolation medium and malonate but not malate was used in the oxygen consumption experiments. The citric acid cycle was inoperative. The oxygen consumption was only due to β oxidation under ketogenic conditions. Since the palmitoylcarnitine and palmitoyl-CoA oxidation capacities were normal, neither the formation of the activated fattyacyl-CoA nor any of the oxidation steps of the palmitoylcarnitine could have been inhibited. These results differ from those experiments in which the citric acid cycle is not inhibited (Fig. 1 and 2). With complete oxidation to CO a significant decrease was observed in the fatty acid oxidizing capacity possible due to a decline in the capacity of the mitochondrial respiratory chain to oxidize the reducing equivalents generated by the citric acid cycle, the latter being apparent only under conditions of high rates of cycle turnover.

Mitochondrial flavins. Mitochondria contain at least three distinct flavin entities, FAD and FMN in the acid-extractable fraction and non-fluorescent flavin in the acid non-extractable fraction. All these flavins were determined from liver mitochondria after severe seven-day hypoxia. The FAD concentration was 0.43 ± 0.02 µmol/g mitochondrial protein in the control group and 0.39 ± 0.06 µmol/g mitochondrial protein in the hypoxic group, the respective FMN concentrations being 0.09 ± 0.01 and 0.10 ± 0.02 µmol/g protein and those of non-fluorescent flavins (like succinate dehydrogenase) 0.15 ± 0.02 µmol/g protein …16 ± 0.02 µmol/g protein. All the results are means from 4 or 5 determinations, and

Results and Discussion

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Uncoupling of mitochondria isolated from fat-containing tissues can be prevented by including albumin in the isolation medium (Martin and Denton 1971), and thus, as an accumulation of triglycerides in the liver was observed in this study (see below), albumin was also used in some of the expts the final concentration in the isolation medium of liver mitochondria being 2%

Fatty acid oxidizing capacity From Fig. 1 and 2 it can be observed that under conditions allowing complete oxidation of fatty acids to CO and water the rate of oxidation in hypoxia declined systematically this effect being most prominent after seven days at 40.8 kPa. The effect of albumin treatment on fatty acid oxidation under non-ketogenic conditions was not tested. The direction of the changes was the same for all the fatty acids studied, *i.e* palmitate

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	B subtilis alkalase	Alkalase omitted
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Palmitoylcarnitine, State 3	116.1±12.4 (5)	106.0±15 9 (5)

* p<0 001

TABLE III. Oxidation of palmitoylcarnitine, palmitate + carnitine and palmitoyl-CoA + carnitine after severe hypoxia at 40 8 kPa for 7 days in albumin-treated rat liver mitochondria under ketogenic conditions. The data are presented as µatoms/min/g protein. Values are means ± S D from the number of expts. indicated in parentheses. The mitochondrial suspension was added to an air-saturated medium consisting of 80 mM KCl, 20 mM Tris-Chloride pH 7 4, 5 mM phosphate, 1 mM EDTA, 5 mM malonate and 2 mM ADP Palmitate was added at the beginning of the expt and the rate of oxygen consumption recorded, after which carnitine was added (2 min later) and the rate of oxygen consumption measured again. Oxidation of palmitoylcarnitine was measured in separate expts.

	Control	Hypoxic
Palmitoylcarnitine	6 40 ± 1.44 (8)	6 63 ± 1 00 (8)
Palmitate	3 30 _ 0 75 (8)	3 01 ± 0 72 (8)
carnitine	4 80 ± 0 95 (6)	4 92 ± 0 70 (6)
Palmitoyl-CoA	3.36 ± 0 82 (6)	3 69 ± 0 60 (6)
carnitine	4.26 ± 0 76 (6)	4 42 ± 0.74 (6)

adaptation is usually impossible, decreased activities are obtained (Ziegler 1967 Kim and Han 1969 Gold and Costello 1974).

The effect of food restriction on mitochondrial respiration was studied as food consumption has been observed to decline in hypoxia. The weight loss of the controls on the restricted diets and the animals undergoing severe hypoxia was the same, about 20 %. State 3 resperation rates and respiratory control ratios of liver mitochondria isolated from the animals on food restriction decreased from the normal 5.8 to 3 4 with succinate, from 6.6 to 3.6 with 2-oxoglutarate and from 5.8 to 3.8 with glutamate + malate (values are means from 3 determinations). State 4 respiration remained unchanged. In this respect the hypoxic and fasting groups show quite similar results, though the decrease in the respiratory control ratios was smaller after fasting.

TABLE IV Effect of hypoxia on the oxidation of succinate by liver mitochondria and the respiratory control ratios (RC) using succinate or glutamate + malate as substrate (no albumin in the isolation medium) The data are presented as µatoms/min/g protein. Values for succinate are means ± S D from 6–8 determinations Respiratory control ratios for glutamate + malate are means from 3 determinations.

		Succinate			Glutamate + malate
		State 4	State 3	RC	RC
Control					
1 day	1	10 6 ± 2 8	52.9 ± 15.2	5 2 ± 0 5	6.3 ± 0.9
7 days	2	10 5 0 7	46 5 ± 11 6	4 4 ± 0.5	5 4 ± 1 1
1 day	3	10 7 ± 2.2	51 7 ± 14 7	4 9 ± 0 6	5 8 ± 0.8
7 days	4	10 1 ± 1 6	45 5 ± 9 9	4 9 ± 0 5	5.3 ± 0 6
50 5 kPa					
1 day	1	12 0 _ 2 0	55 2 ± 14 8	5 1 ± 0 5	5 3 ± 0 6
7 days	2	11 9 ± 2 0	58.5 ± 10 8	5 0 ± 0 8	5.7 ± 0 7
40 8 kPa					
1 day	3	11 7 ± 1.9	45 9 ± 4.7	3.7 ± 0 7[a]	3 9 ± 0 3
7 days	4	12 0 ± 1 1	23 8 ± 7 1	1 9 ± 0 9[b]	3 1 ± 0 2

[a] b. $p < 0\ 001$.

TABLE II Oxidation of palmitate, palmitoylcarnitine and octanoate by liver and heart mitochondria of control rats. Values are means ± S.D from the number of expts. indicated in parentheses. The data are presented as μatoms/min/g protein (22 C). Initially the mitochondrial suspension was added to an air-saturated medium consisting of 80 mM KCl 20 mM Tris-chloride pH 7.4, 5 mM malate 5 mM phosphate 1 mM EDTA and 2 mM ADP Palmitoylcarnitine was added 2 min after the addition of palmitate.

	Heart	Liver
Palmitate	43 1±9.5 (18)	11.5±3.6 (18)
Palmitoylcarnitine	73.2±9 0 (18)	29 3±4 0 (18)
Octanoate	34.5±1.9 (18)	13 9±2.0 (18)

hypoxia induced differences were not statistically significant. These results are in full agreement with the results of unchanged fatty acid β oxidation stated above Any large increase or decrease in the amounts of fatty acid oxidizing enzymes which include two flavoproteins (the acyl-CoA dehydrogenase and the electron transferring flavoprotein) would be probably reflected in an increase or decrease in the total flavin concentration of the tissues, as observed previously (Hassinen and Kähönen 1974) In intact tissues and mitochondrial suspensions, fatty acid oxidation results in extremely large changes in the fluorescence and absorbance of flavins (Hassinen and Hiltunen 1975 Garland *et al* 1967), which emphasize the role of the flavins.

Respiratory control ratio No changes in State 4 or State 3 (Chance and Williams 1955) oxidation rates of glutamate + malate and succinate were induced by moderate hypoxia, nor was any significant change in State 4 respiration observed with either substrate in severe hypoxia. In severe hypoxia, a significant decrease in respiration of liver mitochondria in State 3 was observed (Table IV). In these expts albumin was not used in the isolation medium. The results for the heart mitochondria are shown in Table V In this tissue also State 3 respiration rate was significantly reduced in severe hypoxia.

The reduced respiratory control ratios were not due to uncoupling of the mitochondria since State 4 respiration rates remained almost unchanged It is more likely that the decrease in State 3 respiration results from inactivation of succinate dehydrogenase, or adenylate translocase or the reduced capacity of the respiratory chain proper This interpretation is also supported by the finding that this difference can only be demonstrated under conditions of high turnover in the respiratory chain The decrease in the respiration rate with succinate in severe hypoxia after seven days was statistically significant whereas in moderate hypoxia a slight increase in State 3 respiration occurred (+26) indicating increased succinate dehydrogenase activity (Table IV) There is still great variation in the reports on the effect of hypoxia on succinate dehydrogenase activity One reason is undoubtly the diversity of the assay conditions in different laboratories and the activation–inactivation phenomenon of succinate dehydrogenase *in vitro* It is also known that the activity of succinate dehydrogenase undergoes short-term modulation independently of enzyme synthesis (Aithal and Ramasarma 1969). In most studies increased succinate dehydrogenase activities have been observed in moderate hypoxia (Aithal and Ramasarma 1969 Susheela and Ramasarma 1973a 1973b Sherzer and Cascarano 1972, Kinnula 1975), whereas ere hyp

TABLE III. Oxidation of palmitoylcarnitine, palmitate + carnitine and palmitoyl-CoA + carnitine after severe hypoxia at 40.8 kPa for 7 days in albumin-treated rat liver mitochondria under ketogenic conditions. The data are presented as μatoms/min/g protein. Values are means ± S.D. from the number of expts. indicated in parentheses. The mitochondrial suspension was added to an air-saturated medium consisting of 80 mM KCl, 20 mM Tris-Chloride pH 7.4, 5 mM phosphate, 1 mM EDTA, 5 mM malonate and 2 mM ADP. Palmitate was added at the beginning of the expt. and the rate of oxygen consumption recorded, after which carnitine was added (1 min later) and the rate of oxygen consumption measured again. Oxidation of palmitoylcarnitine was measured in separate expts.

	Control	Hypoxic
Palmitoylcarnitine	6.40 ± 1.64 (8)	6.63 ± 1.00 (8)
Palmitate	3.30 ± 0.73 (8)	3.01 ± 0.77 (8)
carnitine	4.80 ± 0.95 (6)	4.92 ± 0.70 (6)
Palmitoyl-CoA	3.36 ± 0.82 (6)	3.49 ± 0.40 (6)
carnitine	4.26 ± 0.76 (6)	4.42 ± 0.74 (6)

adaptation is usually impossible, decreased activities are obtained (Ziegler 1967; Kim and Han 1969; Gold and Costello 1974).

The effect of food restriction on mitochondrial respiration was studied as food consumption has been observed to decline in hypoxia. The weight loss of the controls on the restricted diets and the animals undergoing severe hypoxia was the same, about 20 %. State 3 respiration rates and respiratory control ratios of liver mitochondria isolated from the animals on food restriction decreased from the normal 5.8 to 3.4 with succinate, from 6.6 to 3.6 with 2-oxoglutarate and from 5.8 to 3.8 with glutamate + malate (values are means from 3 determinations). State 4 respiration remained unchanged. In this respect the hypoxic and fasting groups show quite similar results, though the decrease in the respiratory control ratios was smaller after fasting.

TABLE IV. Effect of hypoxia on the oxidation of succinate by liver mitochondria and the respiratory control ratios (RC) using succinate or glutamate + malate as substrate (no albumin in the isolation medium). The data are presented as μatoms/min/g protein. Values for succinate are means ± S.D. from 6–8 determinations. Respiratory control ratios for glutamate + malate are means from 3 determinations.

		Succinate			Glutamate + malate
		State 4	State 3	RC	RC
Control					
1 day	1	10.6 ± 2.8	52.9 ± 15.2	5.2 ± 0.5	6.3 ± 0.9
7 days	2	10.5 ± 0.7	46.5 ± 11.6	4.4 ± 0.5	5.4 ± 1.1
1 day	3	10.7 ± 2.2	51.7 ± 14.7	4.9 ± 0.6	5.8 ± 0.8
7 days	4	10.1 ± 1.6	43.5 ± 9.9	4.5 ± 0.5	5.3 ± 0.6
50.5 kPa					
1 day	1	12.0 ± 2.0	55.2 ± 14.8	5.1 ± 0.5	5.5 ± 0.6
7 days	2	11.9 ± 2.0	58.5 ± 10.8	5.0 ± 0.8	5.7 ± 0.7
40.8 kPa					
1 day	3	11.7 ± 1.9	45.9 ± 4.7	3.7 ± 0.7[a]	3.9 ± 0.3
7 days	4	12.0 ± 1.1	23.8 ± 7.1[b]	1.9 ± 0.9[b]	3.1 ± 0.2

[a] $p < 0.05$. [b] $p < 0.001$

TABLE V Effect of hypoxia on the oxidation of succinate by heart mitochondria and the respiratory control ratios (RC) using succinate or glutamate + malate as a substrate. The data are presented as μatoms/min/g protein. Values for succinate are means ± S.D. from 6–8 determinations and values for glutamate + malate means ± S.D. from 3–4 determinations.

		Succinate			Glutamate + malate
		State 4	State 3	RC	RC
Control					
1 day	1	33.8 ± 6..	127.4 ± 18.1	4.0 ± 0.4	4.0 ± 0.5
7 days	2	26.6 ± 5.8	112.5 ± 34.9	3.9 ± 0.5	3.8 ± 0.4
1 day	3	31.8 ± 10.7	115.3 ± 12.4	4.0 ± 0.5	3.9 ± 0.4
7 days	4	.5.8 ± 5.1	105.3 ± ?0.2	3.7 ± 0.5	4.0 ± 0.4
50.5 kPa					
1 day	1	34.7 ± 5.8	1.3.1 ± 13.9	3.7 ± 0.4	3.9 ± 0.3
7 days	2	26.2 ± 11.1	146.9 ± 15.3	5.1 ± 0.2	4.0 ± 0.2
40.8 kPa					
1 day	3	26.0 ± 1.5	101.8 ± 18.2	3.9 ± 0.9	3.4 ± 0.5
7 days	4	28.7 ± 6.3	72.8 ± 10.2[a]	2.9 ± 0.6[b]	3.4 ± 0.3

[a] $p<0.01$ [b] $p<0.05$

When the liver mitochondria were prepared in the presence of albumin a slight increase in State 4 respiration was observed (Table VI). This indicates a small degree of uncoupling such as is not apparent in mitochondria prepared without albumin and containing exogenous fatty acids. The expts. on the effects of the uncoupler Cl-CCP on the mitochondria proved unreliable in the presence of albumin because of the variable binding of the uncoupler to the traces of albumin present (data not shown)

There is evidence that fatty acids induce swelling in mitochondria and inhibit some mitochondrial enzyme activities, *e.g.* citrate synthase (Tubbs 1963 Wieland and Weiss 196… The albumin requirement for the isolation of mitochondria of good quality from the liver of hypoxic animals indicates that the properties of these mitochondria are partly due to increased free fatty acid concentration in the liver cells. In experiments on hypoxic mitochondria isolated without albumin, State 3 respiration was markedly lower without much change in State 4 respiration compared with normoxic conditions. This means that the effect is not simply due to an uncoupling, for if this were the case the decline in the respiratory control ratios in the hypoxic mitochondria would result in an increase in State 4 respiration, not a decrease in State 3 respiration. Reduced respiratory control ratios and slower State 3 respiration rates have also been found when mitochondria are isolated without albumin from perfused ischemic liver (Bolme *et al.* 1970) In that case the decline in the respiratory control ratios parallels the rise in the free fatty acids and decrease in cardiolipin in the mitochondria.

Triglycerides A significant accumulation of liver triglycerides occurred during the severe 7-day hypoxic period (Table VII) though moderate hypoxia does not cause any deposition of triglycerides. The origin of the fatty acids in hypoxia-induced triglyceride accumulation is uncertain but lipolysis from the adipose tissue may partly contribute to it. Serum free fatty acid levels were unchanged after seven days in severe hypoxia (Table VII), but their concentration had increased by 22% after one day under the same conditions (data not sho…

TABLE VI Effect of hypoxia for 7 days at 40.8 kPa on the oxidation of succinate, 2-oxoglutarate and glutamate + malate by liver mitochondria with albumin added to the isolation medium. The data are presented as μatoms/min,g protein. Values are means ± S.D. from the number of experiments indicated in parentheses.

	State 4	State 3	RC
Succinate			
Control	7.4 ± 1.1 (10)	41.2 ± 7.1 (10)	5.5 ± 1.2 (10)
Hypoxic	11.0 ± 3.4[a] (10)	42.6 ± 7.4 (10)	4.2 ± 0.9[b] (10)
2-Oxoglutarate			
Control	2.6 ± 0.7 (5)	14.1 ± 5.0 (5)	5.2 ± 1.1 (5)
Hypoxic	2.9 ± 0.8 (5)	12.5 ± 2.6 (5)	4.7 ± 2.2 (5)
Glutamate + malate			
Control	3.4 ± 1.1 (5)	19.4 ± 3.8 (5)	6.6 ± 2.2 (5)
Hypoxic	3.0 ± 1.6 (5)	17.2 ± 4.1 (5)	5.9 ± 2.1 (5)

p 0.002 p 0.001

Reduced fatty acid oxidizing capacity however probably offers a more plausible explanation for the development of the hepatic steatosis. In this respect the mechanism may be similar to the development of ethanol-induced fatty liver which seems to be due mainly to an inhibition of hepatic fatty acid oxidation with a simultaneous activation of the fatty acid esterification pathways (Blomstrand and Kager 1974). It has been shown that even moderate hypoxia increases sympathoadrenal activity (Euler and Hellner 1952, Surks *et al.* 1967). Hypoglycemia has also been reported in hypoxia (Timiras *et al.* 1958) and prolonged hypoxia is known to result in a depletion of hepatic glycogen (Timiras *et al.* 1958, Gibbs and Gold 1971). These are changes which are likely to exercise an adverse effect on lipogenesis in the liver. It has also been reported both with perfused livers (Salmon *et al.* 1974) and *in vivo* (Savolainen *et al.* 1977) that situations of carbohydrate depletion coincide with diminished fatty acid synthesis. Lactate can in perfused liver control lipogenesis by substrate limitation

TABLE VII Effect of 7-day hypoxia and food restriction (pellets for 1h/day) on tissue triglyceride concentrations and serum free fatty acid levels. Concentrations are expressed as μmol/g wet weight for triglycerides and μmol/ml for serum free fatty acids. Values are means ± S.D. from the number of expts. indicated in parentheses.

	Triglycerides		Free fatty acids
	Heart	Liver	Serum
Control			
1	3.6 ± 1.0 (3)	6.6 ± 1.5 (4)	0.49 ± 0.08 (3)
2	3.9 ± 1.7 (7)	7.3 ± 1.1 (12)	0.61 ± 0.11 (10)
3	—	6.5 ± 1.5 (4)	0.63 ± 0.13 (4)
Hypoxic			
50.5 kPa 1	3.3 ± 0.9 (3)	4.5 ± 0.6 (4)	0.61 ± 0.11 (3)
40.8 kPa 2	3.0 ± 0.7 (7)	23.3 ± 14.2[a] (12)	0.59 ± 0.22 (10)
Food-restricted			
controls 3	—	2.3 ± 0.4[a] (4)	0.86 ± 0.21 (4)

(Salmon *et al* 1974) Increased liver lactate concentrations occur in hypoxia (Cipriano & Pace 1971) but it does not seem probable that the lactate control of lipogenesis is able to override the lipolytic effects of the sympathoadrenal activity

The technical assistance of Mrs Raija Sirviö and Mrs Katriina Kippo is gratefully acknowledged

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From the Institute of Physiology University of Helsinki, Finland

Efflux of phenylalanine and tryptophan from cerebral cortex slices of adult and 7-day-old rats

By

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Abstract

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An equation of two exponential terms was found to adequately describe the efflux of phenylalanine tryptophan from cerebral cortex slices f adult nd 7-day-old rats. These exponential terms described forms (components) of efflux taki g place at d fferent rates. The fast component of efflux was more pro inent in the slices from young rats than in those from adults. I both age groups the contribution of t fast component increased with an increasing amino acid concentration in the superfusate but there were changes in the rate constants. The rate constants of the fast components were equal in ll experiment conditions in both age groups. The rat constants for the slow components of both amino acids ere low in the slices from adult rats than n those from young rats. The reason for efflux occurring at two differ rates is discussed. The fast component probably consisted of the amino acid originati g from the ex cellular space of the slices nd of the intracellula amino acid released by exchange The slow compon consisted of the amino acid released from the cells by other mechanisms, e g. by d ffusion through membranes or by some active efflux processes. The cerebral cortex slices from adult rats have grea ability to concentrate aromatic amino acids than the slices from young rats. This may partly be depend on the more effecti influx and partly o the slower efflux of amino cids in the slices from adults

Key ords: Cerebral cortex slices, brain development, transport of amino acids, phenylalanine, tryptoph

The properties of amino acid influx into cerebral slices have been much studied (Oja a Vahvelainen 1975) Some of these studies have concerned changes in the influx duri ontogenetic development (Vahvelainen and Oja 1972, Vahvelainen and Oja 1975) Less known about the mechanism of amino acid efflux from the slices and about its possib changes during development.

The efflux of amino acids from adult nervous tissue is assumed to be at least partly a active process it is saturable in some cases, stereospecific dependent on temperature an ion concentrations in the medium there is competition among amino acids transported from the cells, and the efflux rate can be modified by compounds influencing cell metabolis (Levi *et al* 1965 Levi *et al* 1966 Cherayil *et al* 1967 Srinivasan *et al* 1969 Blasberg *et al* 1970 Jones and Banks 1970 Arnfred and Hertz 1971 Cutler *et al* 1971 Hopkin 1971 Joanny *et al* 1971 Joanny *et al* 1973 Mulder and Snyder

In addition to possible active efflux mechanisms and simple diffusion, a process called exchange may contribute to the efflux of amino acids. Both homoexchange (transport stimulated by the presence of the same amino acid on both sides of the membrane) and heteroexchange (transport stimulated by the presence of structurally related amino acids on the two sides of the membrane) have been demonstrated in brain slices (Joanny *et al.* 1971 Cutler *et al.* 1971 Battistin *et al.* 1972, Joanny *et al.* 1973).

This paper reports on a study of the efflux of phenylalanine and tryptophan from the cerebral cortex slices of adult and 7-day-old rats and its enhancement by homoexchange. These amino acids were selected for study for the following reasons. 1) The transport of aromatic amino acids is especially interesting in nervous tissue because of their role as precursors of biogenic amines. 2) Since there are differences in the influx of these amino acids into cerebral cortex slices prepared from adult and young rats (Vahvelainen and Oja 1972), it is possible that also the efflux of these amino acids changes during development. 3) Phenylalanine and tryptophan compete in influx (Vahvelainen and Oja 1975) indicating that they at least partly share the same transport mechanism. The efflux mechanisms of these amino acids may therefore also be similar

Materials and Methods

The radioactive amino acids -tryptophan-H^3 (G) (sp. act. 1 0 Ci/mmol) and 3-phenyl(alanine-2,3-H^3) (sp. act. 5 0 Ci/mmol) were purchased from the Radiochemical Centre, Amersham. Slices of cerebral cortex were prepared from 7-day-old (10–15 g) and adult (180–200 g) Sprague-Dawley rats. The animals were decapitated, their brains excised and briefly rinsed in cold 0 9 saline. The slices were cut at 4°C with non-wetted razor sheer of Stadie-Riggs type. The thickness of the slices was 0 5 mm, one side of each slice was the intact brain surface

Experimental procedure

In each experiment 6 slices weighing in total about 300 mg were preincubated in an Erlenmeyer flask for 10 min under continuous flow of pure oxygen at 37°C in Warburg apparatus and shaken (80 oscillations/min, amplitude 20 mm) The medium was 5 ml Krebs-Ringer phosphate solution (pH 7 4) (Umbreit *et al.* 1964) (final concentrations in mmol/l: NaCl, 127 KCl, 3.1 $CaCl_2$, 2.7 KH_2PO_4, 1.3 $MgSO_4$, 1.3 and Na_2HPO_4–HCl-buffer 10) containing 10 mmol/l glucose, 0 5 mmol/l unlabelled tryptophan or phenylalanine, and mCi l of the corresponding tritiated amino acid. The medium was prewarmed and preoxygenated for 15 min before immerging the slices.

The slices were separated from the preincubation medium by filtering and rinsed briefly with 15 ml of cold Krebs-Ringer phosphate solution. Then they were transferred rapidly one at a time into small glass cylinders. The cylinders contained about 2 ml of superfusion medium: Krebs-Ringer phosphate solution with glucose (10 mmol/l) and unlabelled phenylalanine or tryptophan (0 05, 0 5 or 5 0 mmol/l). Two slices were put into each cylinder. These incubation vessels had been previously connected to an electromechanic pumping apparatus (Desaga, Heidelberg) (Fig. 1). The superfusion medium in the inlet reservoirs, in the silicone tubes and in the incubation cylinders was prewarmed and preoxygenated by bubbling for 30 min. The flow rate of the medium was adjusted to about 1 ml/min. It was measured during 15 min before and after every incubation and varied from 0 9–1.3 ml/min. During the incubation it did not change more than 0 1 ml/min.

The efflux of radioactivity out of the slices was followed for 60 min. The effluents were collected manually from the outlet tubes directly into scintillation counting vials which were changed every 2 min. The volumes of the fractions were calculated from the measured flow rates of the superfusates. The average fraction volume was 2.3 ml (range 2 07–2 60 ml). During incubation the slices were oxygenated, thermostated and shaken in the Warburg apparatus as during preincubation. At the end of incubation the volumes of the medium in the incubation vessels and in the outlet tubes were measured.

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Abstract

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An equation of two exponential terms was found to adequately describe the efflux of phenylalanine and tryptophan from cerebral cortex slices of adult and 7-day-old rats. These exponential terms described two forms (components) of efflux taking place at different rates. The fast component of efflux was more prominent in the slices from young rats than in those from adults. In both age groups the contribution of the fast component increased with an increasing amino acid concentration in the superfusate, but there were no changes in the rate constants. The rate constants of the fast components were equal in all experimental conditions in both age groups. The rate constants for the slow components of both amino acids were lower in the slices from adult rats than in those from young rats. The reason for efflux occurring at two different rates is discussed. The fast component probably consisted of the amino acid originating from the extracellular space of the slices and of the intracellular amino acid released by exchange. The slow component consisted of the amino acid released from the cells by other mechanisms, e.g. by diffusion through the membranes or by some active efflux processes. The cerebral cortex slices from adult rats have greater ability to concentrate aromatic amino acids than the slices from young rats. This may partly be dependent on the more effective influx and partly on the slower efflux of amino acids in the slices from adults.

Key words: Cerebral cortex slices, brain development, transport of amino acids, phenylalanine, tryptophan

The properties of amino acid influx into cerebral slices have been much studied (Oja and Vahvelainen 1975). Some of these studies have concerned changes in the influx during ontogenetic development (Vahvelainen and Oja 1972, Vahvelainen and Oja 1975) Less is known about the mechanism of amino acid efflux from the slices and about its possible changes during development.

The efflux of amino acids from adult nervous tissue is assumed to be at least partly an active process it is saturable in some cases, stereospecific, dependent on temperature and ion concentrations in the medium, there is competition among amino acids transported from the cells, and the efflux rate can be modified by compounds influencing cell metabolism (Levi *et al.* 1965 Levi *et al* 1966, Chernyll *et al* 1967 Srinivasan *et al* 1969 Blasberg *et al.* 1970 Jones and Banks 1970 Arnfred and Hertz 1971 Cutler *et al* 1971 Hopkin and Neal 1971 Joanny *et al.* 1971 Joanny *et al* 1973 Mulder and Snyder 1974

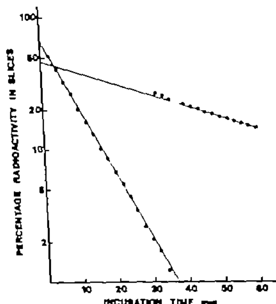


Fig. 2. Method of analysing the efflux curve. The mean values of 6 expts. of [³H]phenylalanine efflux from cerebral cortex slices prepared from adult rats and superfused with medium containing 5.0 mmol/l unlabelled phenylalanine are presented as an example (●) Experimental results, per cent of initial radioactivity left in the tissue on a logarithmic scale plotted against time after beginning washout. After about 40 min the points can be considered to fall on a straight line. The slow component of the curve is obtained from this line. The fast component (○) is obtained by subtracting the line from the experimentally determined points. The values of the fast component can also be considered to fall on a straight line. The slopes of the two lines represent the rate constants for the two components. The extrapolation of the two lines to time zero gives the percentages of the total initial radioactivities of the components. The final analyses were computed separately for each individual expt. Overall means and standard deviations were also computed. See text for more details.

where f is the radioactivity in the slices (per cent of the total initial radioactivity) at time t (min), F and F are the total initial radioactivities in the two components of efflux (per cent of the total initial radioactivity in the slices), and k and k_2 are the rate constants (min^{-1}) for the amino acid efflux in the two components. The values recorded during the first 4 min were omitted from the calculations because of the dilution of the released label in the nonradioactive superfusion medium initially present in the outlet tubes (Fig. 3). The total radioactivity in the slices at 4 min was taken as 100 per cent.

The final analyses of the curves were made for each individual expt. in the following way (see Fig. 2). First the slow component was determined by calculating the linear correlation between the incubation time and the logarithmic value of percentage radioactivity left in the slices for the last 5 measurements (at 52, 54, 56, 58 and 60 min). Earlier measurements were then added one by one to the regression data until the absolute value of the correlation coefficient decreased two times successively. The two poorly fitting values were then discarded from the data and a least-squares line was fitted through the remaining points. The average number of points remaining in the slow component was eight, and the correlation coefficients varied from −0.99999 to −0.99483. The slow component was then subtracted from the experimental values and another least-squares line was fitted through the remaining points to obtain the regression for the fast component. The correlation coefficients for the fast-component lines varied from −0.99915 to −0.96465. The slopes of the lines represent the rate constants for the two components of efflux (k and k_2). The extrapolation of the lines to time zero gives the total initial radioactivities in the two components (F and F_2). The sum of F and F was in all cases a little over 100 per cent (about 105 per cent). This was interpreted to be because the calculations were not iterated leaving some of the fast component in the slow component. … the errors were small, the iterations were considered unnecessary and the values obtained by

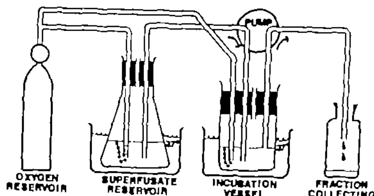


Fig. 1 The experimental [...] for the study of amino acid [...] from tissue slices by continu[...] washout.

After incubation the slices were separated by filteri g, weighed in 1.0 ml of 5% trichloroacetic [...] (TCA) (w/v) homogenized and centrifuged for 30 min at 10 000 g. The radioactivity of the TCA-su[...]natants, of the effluent fractions, of the samples from the superfusion medium left in the outlet tubes [...] of the samples from the preincubation med um was measured by liquid scintillation counting ([...] NTL) in Aquasol® (New England Nuclear Dorval, Quebec). The quenching of the samples was de[...]mined by the external standard channel ratio method and the efficiency of counting from standard samp[...]

Chromatographic analyses

In order to check the possible metabolic breakdown of the labelled phenylalanine and tryptophan du[...] the expts. number of slices were preincubated as above for 30 min with 10 mCi/l [³H]tryptophan [...] [³H]phenylalanine. Some slices were then superfused for 60 min as bove. Each experiment as repea[...] 4 times. After the incubations the slices were immediately homogenized with 0.5 ml of cold 75 etha[...] containi g 0.05% edetat and 0.05% ascorbic acid. The homogenates were centrifuged for 30 min [...] 10 000 g stored overnight at +4°C nd centrifuged again. Samples of 10 μl from the supernatants w[...] applied on 0.25 mm silica gel thi layer plates (Merck AG) and developed w th phenol–water (3 1 [...] and n-butanol–ethanol–25% ammonia (8 1 1 v/v/v). Standard amino acid mixtures were run in paral[...] and stai ed with ninhydrin o Ehrlich reagent in order t localize the spots correspond ng to phenylala[...] nd tryptophan in the unstained xperimental samples. The portion of the gel conta ning the chromat[...]graphed samples was scratched out in small spots which were suspended i 94% ethanol and mixed w[...] Instagel and counted with the liquid scintillation spectrometer Quenching nd efficiency of count[...] wer determined as above.

On the average, in preincubated but not superfused slices 89 per cent of the original radioactivity w[...] recovered as [³H]tryptophan and 96 per cent as [³H]phenylalanine representing more tha 91 and 96 per ce[...] of the total radioactivity recovered from the thin layer plates, respectively I preincubated and superfu[...] slices 87 per cent of the original radioactivity was recovered as [³H]tryptophan and 98 per cent as [³H]phen[...]alanine representing more than 96 and 98 per cent of the total radioactivity recovered after chromatograph[...] Standard deviations in these analyses varied from 1 t 4 per cent. Labelled phenylalanine and tryptoph[...] were declared chromatographically 100 pure by the manufacturer no radioacti e impurities ere d[...]tected by us either Measurable amounts f radioacti metabolites were not found in the effluents co[...]lected from the incubation vessels. These checks do not show any major breakdown of the labelled amin[...] acids during the efflu experiments.

Calculations

The initial radioactivity in the slices was computed as the sum of the radioactivities i the effluent fractio[...] in the medium left i the outlet tubes and in the TCA-sample (the residual tissue radioactivity except th[...] label in the TCA-precipitates). The percentage of the label remaining in the tissue at ny time was calcula[...] by subtracting the total radioactivity recovered in the effluent from the initial tissue content.

The concentration of the mino acid in the slices before w shout was calculated from the initial rad[...]activity by assumi g that the intracell lar amino acid pool were homogeneously l belled d ring the pre[...]incubation and ignoring y metabolic breakdown of the labelled amino cids

According to preliminary graphic analyses (F g. 2 is an example) the efflux of the label seemed to b[...] satisfactorily described by the follow ng equation (g Bradl g 1971)-

$$f_t = F_1 \quad ^{k\ t} + F \quad ^{k} \qquad 1$$

Table I Concentrations of labelled phenylalanine and tryptophan in the cerebral cortex slices of rats before washout, and radioactivity left in the slices after washout.

Age of animals	Amino acid and its conc. in superfusion medium (mmol/l)		Conc. of amino acid before wash-out (μmol/kg)	Radioactivity left after wash-out (%)
Adult	Phenylalanine	5.0	568 ± 57 (18)	14 ± 3 (6)
		0.5		17 ± 2 (6)
		0.05		23 ± 2 (6)
	Tryptophan	5.0	573 ± 87 (16)	17 ± 4 (6)
		0.5		23 ± 2 (5)
		0.05		31 ± 7 (5)
7-day-old	Phenylalanine	5.0	654 ± 56 (17)	5 ± 2 (6)
		0.5		5 ± 2 (6)
		0.05		10 ± 2 (5)
	Tryptophan	5.0	669 ± 57 (18)	6 ± 1 (6)
		0.5		10 ± 2 (6)
		0.05		16 ± 2 (6)

The slices were preincubated for 30 min at 37°C in oxygen in Krebs-Ringer phosphate medium (pH 7.4) containing glucose (10 mmol/l), [^{3}H]amino acid (2 mCi/l) and unlabelled amino acid (0.5 mmol/l). The concentrations of the amino acids in the slices with their standard deviations are expressed as μmol/kg of the fresh weight of tissue. The release of radioactivity into flowing superfusion medium containing varying concentrations of the corresponding unlabelled amino acid was sampled for 60 min. The radioactivities left in the slices after washout were measured, and their means and standard deviations are expressed as percentages of the initial radioactivities. Number of experiments shown in parentheses.

of the components were about equal for both amino acids in the corresponding concentrations. At the concentration of 5.0 mmol/l the slow component contained slightly more than half, at the concentration of 0.05 mmol/l about 80 per cent of the total radioactivity washed out. In the slices from 7-day-old rats the share of the slow component was generally less than in those from adults. When the amino acid concentration in the superfusate was low some difference between the amino acids was found in the slices from young rats: more tryptophan (81 per cent) than phenylalanine (53 per cent) flowed within the slow component (Table II).

Table II Percentages of the total initial radioactivities released in the slow (F_1) and fast (F_2) components of amino acid efflux from cerebral cortex slices prepared from adult and 7-day-old rats.

Amino acid and its conc. in superfusion medium (mmol/l)		Adult rats		7-day-old rats	
		F ( )	F ( )	F ( )	F ( )
Phenylalanine	5.0	54 ± 2	46 ± 3 (6)	33 ± 8	67 ± 8 (5)
	0.5	67 ± 5	33 ± 8 (6)	40 ± 5	60 ± 10 (3)
	0.05	77 ± 3	23 ± 2 (6)	53 ± 3	47 ± 4 (5)
Tryptophan	5.0	54 ± 3	46 ± 5 (5)	34 ± 6	66 ± 7 (6)
	0.5	66 ± 7	34 ± 12 (5)	52 ± 8	48 ± 10 (6)
	0.05	83 ± 5	17 ± 4 (4)	81 ± 6	19 ± 6 (6)

The slices were preincubated for 30 min with radioactively labelled amino acid (conc. in medium 0.5 mmol/l). Then the release of the radioactivity into flowing superfusion medium containing varying concentrations of the corresponding unlabelled amino acid was measured in two-minute fractions during 60 min. The mean percentages and their standard deviations were calculated as described in Material and Methods. Number of experiments shown in parentheses.

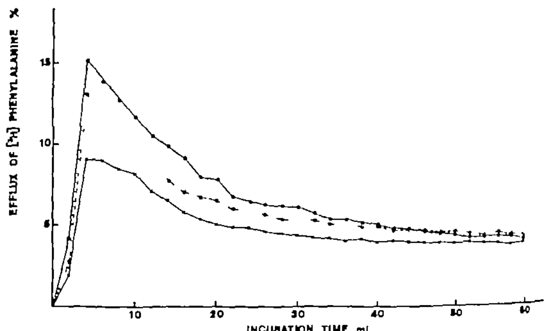


Fig. 3 The efflux of [³H]phenylalanine from cerebral cortex slices prepared from adult rats and superfused with media containing 5.0 (●—●), 0.5 (○—○) or 0.05 mmol/l (—×) unlabelled phenylalanine. The slices were preincubated for 30 min at 37°C in oxygen in Krebs-Ringer phosphate solution (pH 7.4) containing glucose (10 mmol/l) [³H]phenylalanine (mCi/l) and unlabelled phenylalanine (0.5 mmol/l). In order to measure the release of radioactivity the slices were then transferred into the each in which they were superfused with medium containing varying amounts of unlabelled phenylalanine. Fractions of about 2 ml were collected every 2 min The results, as means of 6 expts., denote the percentage of the total amount of label in the slices which is released during 2 min i tervals after varying incubation periods. Standard deviations for each point were 5–15% usually not more than 10%.

computatio were proportionally adjusted to add up to 100 per cent. The standard deviations for k k_f F and F were computed fo the slopes nd intercepts of regression lines (e g Snedecor 1959).

The half-time (t1/2) of efflux fo each component was determined from the equation (g Bradley 197

$$t1/2 = \frac{0.693}{k}$$ (2)

where k is the rate constant for the component.

Results

After preincubation the concentrations of phenylalanine and tryptophan were of the same order in the slices from both adult and 7-day-old rats after washout the radioactivity left in the slices was greater in the adult group (Table I) The efflux of both amino acids into the superfusion media containing varying concentrations of these was rather similar in both age groups the higher the amino acid concentration in the superfusate the more rapid was the efflux of the label and the less radioactivity remained in the slices after the washout (Table I Fig. 3)

When the efflux was divided into the two components, it became obvious that the higher the amino acid concentration in the superfusion medium the greater was the contribution of the fast component (Table II), but there were no changes in the rate constants as functions of the amino acid concentration (Table III) In the slices from the adult rats the percentages

d used supposes that both the extra- and intracellular compartments are directly con- with the bulk phase. This is an unrealistic assumption (Jones and Banks 1970). ever the idea that the fast component may partly contain the amino acid originating the extracellular space can be supported by two facts 1) The share of the fast com- nt was greater in the slices from young rats, and the size of the extracellular space is r in the cerebral cortex slices from 7-day-old rats than in those from adult rats (Laakso Oja 1976) 2) The rate constants for all the "fast effluxes" were equal, which can be cted in the case of free diffusion of substances of equal molecular size.

utler *et al.* (1971) have analyzed the efflux of glycine and GABA from spinal cord slices concluded that the fast component of the desaturation curves reflected the washout of erent medium and the slow component the loss of isotopes from the tissue as a whole. s interpretation is probably not valid for the present study because of the relatively rament contribution of the fast component and because we rinsed the slices after pre ubating them in the radioactive amino acid.

Hertz (1968) studied ion transport in brain slices and inferred that the two components of lux in his study originated from different intracellular compartments. If both components this study contain amino acids of intracellular origin, at least two interpretations can be ggested for having two separate components. Firstly the intracellular amino acids may be ated in different *compartments*, from which the efflux occurs at different rates, and sec dly there may be several *processes* in the efflux of amino acids at different rates. The lidity of the compartment hypothesis is supported e.g. by findings that glutamate is ex anged more rapidly in glial cells than in neurons of cerebral slices from rats (Okamoto d Quastel 1972) If the components of efflux in our experiments originated from differen mpartments, raising the amino acid concentration in the medium should enlarge th pidly exchanging compartment. In our view more evidence would be required to suppor is assumption

Explaining the components as representing fast and slow efflux processes allows mor roductive discussion. Because the contribution of the fast process increased with increasin mino acid concentration in the superfusate, it might represent the exchange efflux, and th low process might consist of other efflux mechanisms. If this interpretation is right, th ollowing conclusions can be drawn 1) the exchange efflux compared to the other efflu mechanisms may be more prominent in the slices from young rats 2) the rates of exchang efflux are equal in both age groups, but 3) the other efflux mechanisms are slower in th *slices from adults, 4) the* rates of efflux of phenylalanine and tryptophan by the slow pr cesses are equal in the slices from young rats, but 5) phenylalanine is released more rapid than tryptophan in the slices from adult rats.

The slow efflux may comprise passive diffusion of amino acids out of the cells (witho exchange) and some active efflux processes. It seems improbable that the constants of t slow processes describe only passive diffusion, because there are differences between t age groups and between the amino acids. The differences between the age groups could a be explained by the more efficient re-uptake of the label in the slices from adult rats. A this seems unlikely The re-uptake was minimized by using a relatively high flow rate of superfusion (Raiteri *et al.* 1974) and a large medium-volume/tissue-volume ratio (50

TABLE III Rate constants and half times for slow (k_1 $t1/2_1$) and fast (k_2, $t1/2_2$) components of amino
efflux from cerebral cortex slices prepared from adult and 7-day-old rats.

Amino acid and its conc. in superfusion medium (mmol/l)	Adult rats				7-day-old rats			
	$k \times 10^{-3}$ (min⁻)	t1/2 (min)	$k_2 \times 10^{-3}$ (min)	$t1/2_2$ (min)	k 10⁻³ (min)	t1/2 (min)	k_1 10⁻³ (min⁻¹)	t1 (
Phenylalanine								
5.0	21±3	33	134±7	5.2 (6)	[illegible]6±5	27	150±15	4 6 (
0.5	[illegible]2±2	32	137±9	5 1 (6)	31±4	2[illegible]	141±11	4 9 (
0 05	19±1	36	146±18	4 7 (6)	28±3	25	130±4	5 3 (
Tryptophan								
5 0	16±4	4	142±13	4 9 (5)	24±2	29	141±5	4 9 (
0.5	18±3	39	145±48	4 8 (5)	28±1	[illegible]5	142±13	4 9 (
0.05	14±2	49	147±9	4 7 (4)	6±2	27	149±40	4 7 (

The values of k and k_2 with their standard deviations were calculated for the same expts. as in Tabl
as described in Material and Methods. The values of $t1/2_1$ and $t1/2_2$ were calculated from the rate cons
($t1/2 = 0\ 693/k$) Number of expts. shown in parentheses.

The rate constants of the fast components were of equal magnitude for both amino a in both age groups, but the rate constants of the slow components were smaller in the sl from adults (Table III) (Student s two-tailed t test gave $p < 0\ 01$ in all tryptophan ex and at the concentrations of 0 5 and 0 05 mmol/l in phenylalanine expts.) Between amino acids there were no significant differences in the slices from 7-day-old rats, but in slices from adult rats the rate constants for tryptophan were a little smaller than for phe alanine ($p < 0\ 01$ at the concentration of 0 05 mmol/l and $p < 0\ 05$ at the concentratio 0 5 mmol/l) The calculated half-times for the efflux components show a similar pic (Table III) The half times for all the fast components were about 5 min. The half-times phenylalanine in the slow component were about 34 and 25 min and those for tryptop about 43 and 27 min in the slices from adult and young rats respectively

Discussion

The computed efflux curves were practically identical with the experimental curves. efflux of amino acids from the cerebral cortex slices was thus well described by the equa of two exponential terms. What remains to be clarified is the reason for the two compone Several hypotheses have been previously published mainly suggesting that the compon flow from different sites within the tissue or from its surface

In some discussions mostly concerning the efflux of electrolytes the fast and slow c ponents are considered to be released from extra and intracellular compartments of tiss respectively (Winegrad and Shanes 1962, Zadunaisky and Curran 1963 Ames and Nes 1966, Borle 1969 Kennedy and Voaden 1974). For the data of the present study this in pretation may not be valid for the following reasons 1) Increasing the concentration of unlabelled amino acid in the superfusate relatively increased the recovery of the am acids within the fast component. There is no reason to believe that the extracellular spac the slices enlarged noticeably at higher amino acid concentrations. 2) The mathe a

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Also the fact that the efflux of the label in the slow component did not increase with increasing amino acid concentration in the superfusate, supports the view that the differences in the rate constants were not caused by different rates of re uptake.

In conclusion, the most probable explanation for the two components of efflux in the study is that the fast component consists of the amino acid released from the extracellular space of the slices and the amino acid released from the cells by exchange, and the slow component involves other efflux processes from within the cells.

The equilibrium concentration of an amino acid in the slices depends both on the influx and the efflux. The cerebral cortex slices from adult rats have been found to concentrate *e.g.* tyrosine more effectively than the slices from young rats (Vahvelainen and Oja 1972). The maximum velocity of the saturable influx of aromatic amino acids is greater in the slices from adult rats (Vahvelainen and Oja 1972) The present experiments show that the efflux of these amino acids is more rapid from the slices, and probably from within the cells in the slices from young rats. This cannot be proved by the greater share of the fast component in the slices from young rats, because we do not know how much of it originates in the cells. But the smaller rate constants of the slow components in the slices from adults suggest that the slower efflux contributes to the greater ability of the slices from adults to concentrate aromatic amino acids.

The skilful technical assistance of Mrs Pirkko Erkkilä is gratefully acknowledged. I am also indebted to Mr Esa Korpi, B M for his aid in chromatographic analyses.

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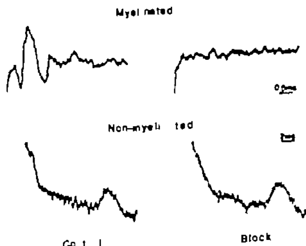
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1 Anodal block of myelinated fibres in the left aortic nerve, as illustrated by its effects on evoked pound action potentials Separate oscilloscope sweeps, all ith the same vertical sensitivity Stimulus characteristics A 0 02 ms, 6 V C 1.5 ms, 6 V Note that compound A aves were absent during block, hile the compound C wave remained unaltered.

n our first study of the function of baroreceptor C fibres, we examined the contribution eft aortic nerve C fibres to pressure-induced bradycardia, by recording changes in heart when the aortic nerve was fully intact and when its A fibres had been temporarily cked by a polarizing current (anodal block). The bradycardia resulting from a moderate in pressure was found to be exclusively mediated by baroreceptor A fibres. a rise in tolic pressure of 20–30 mmHg was insufficient to evoke bradycardia via aortic nerve C res in the rabbit (Aars and Myhre 1974). We now report the results of a more comprehen- study of the function of aortic nerve C fibres, in which the reflex responses to a pressure e were assessed by changes in sympathetic discharge to the kidney As in the earlier report, flexes its aortic nerve A and C fibres were separated by means of anodal block of the A res

Material and Methods

abbits were anaesthetized ith 3 ml 1 chloralose and 3 ml 25 % urethan per kg; half i half i p When ecessary urethan as supplemented through the right jugular vein. Body temperature was maintained means of covers and heating lamp The animals were tracheotomized and respired air Arterial pres- ure as measured ith Statham P23Db transducer connected to catheter in the right common carotid rtery Brief rises of 20–60 mmHg mean pressure in the aortic arch could be induced by inflation of balloon Fogarty Arterial Embolectomy Cath. 4F) inserted through the left femoral artery and positioned in the lescending thoracic aorta. Sympathetic nerve activity was recorded in the left renal nerve with silver lectrodes and differential amplifier (Grass P15 B) The activity was averaged (Coupler 9852 A) and ecorded on Beckman R 411 Dynograph, together with the blood pressure. Output from the averager was inearly related to the activity found in the renal nerve The left aortic nerve was dissected in the neck and overed by paraffin oil Three pairs of silver electrodes (diameter 0 6 mm) ere placed on the left aortic erve for electrical stimulation (Grass SD9) in the caudal part, for anodal block in the middle and cranially or recording of electrical activity in the nerve (Grass P15 B) The anodal block as effected by passing current through the nerve between tw electrodes 3–4 mm apart, the current being delivered from specially f ery-operated constant current generator The slope of the rising current was less than 10 μA/s.

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The function of baroreceptor C fibres in the rabbit's aortic nerve

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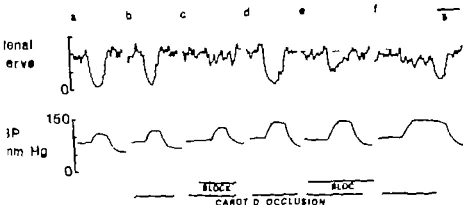
Abstract

AARS, H L. MYHRE and B A. HASWELL. *The function of baroreceptor C fibres in the rabbit aortic nerve* Acta physiol scand 1978 *102* 84–93

The participation of aortic nerve C f bres in the baroreflex was investigated by recordi g changes in renal nerve activity i response to acute increases in arterial pressure, in two experimental situations: with A and C fibres of the left aortic nerve intact, and with th A fibres temporarily blocked by a hyperpolarizing current (anodal block) Pressure was increased by manual inflation of an intra-aortic balloon, and interference from other baroreceptor reas was a oided by carotid occlusion and sectioning of the right aortic nerve. Rises in mea arterial pressure exceeding 20 mmHg, t levels bove 110 mmHg, were needed to trigger sympathetic inhibition via C f bres. A rise of 45 mmHg caused 50 % reduction in sympathetic activity equal to that obtained by stimulation of ortic nerve C fibres at about 3 Hz. In contrast, a rise of 20–30 mmHg evoked more than 60 % reduction in sympathetic activity when the A fibres were operative. Judged by these studies of peak reflex responses to brief pressure rises, baroreceptors with C f bres in the aortic nerve have a much higher threshold to pressure than thei myelinated counterparts the C fibres contribute t the baroreflex nhibition of sympathetic discharge only when pressure is increased well above normal resting levels.

Despite the existence of a high proportion of non-myelinated baroreceptor afferents (C fibres) in the aortic and carotid sinus nerves, the knowledge of baroreceptor function is almost completely restricted to receptors with myelinated (A) fibres (Heymans and Neil 1958 Kirchheim 1976) All that is known of the C fibres, is that when electrically evoked activity in these fibres will induce severe reflex bradycardia, sympathetic inhibition and fall in blood pressure (Douglas and Ritchie 1956, Douglas, Ritchie and Schaumann 1956 Douglas and Schaumann 1956, Kendrick and Matson 1971 Ninomiya, Nishimaru and Irisawa 1971 Koizumi and Sato 1972, Kardon, Peterson and Bishop 1973 1975) Response characteristics of the C fibre receptors, and hence their contribution to the baroreflex, remain virtually unknown.

Preliminary presentations of this paper were given in J ne 1976 t the 7th European Congress of Cardiology Amsterdam, The Netherlands, and at the 8th Nordic Hypertension Meeting, Marstrand (Aars, Myhre and Haswell 1976).



g 3 Reflex responses of average sympathetic activity in renal nerve to balloon-induced rises in arterial ean pressure. Influences from right aortic nerve and right carotid sinus excluded. With left aortic and l sinus nerves intact. b–f Afferent baroreceptor pathways restricted to the left aortic nerv by transitory cclusion of left carotid artery. Responses to moderate (b–c) nd larger (d–e) rises of pressure. In periods l nodal block (c), aortic baroreceptors were functionally connected to the brain through aortic C bres only barces in records b d both A and C fibres could transmit action potentials. The figure shows ia the threshold for excitation of receptors ith aortic C fibres was exceeded in records d but not bardy reached by the pressure rises in b Record f show the obligatory test ending all experiments: e recording of sympathetic effects of pressure rise after the left aortic nerve had been cut. A slight reponse persisted, which was subsequently found to result from excitation of gal afferents. The sharp nhibition caused by activation of carotid sinus receptors (release of carotid occlusion) served as final heck on the integrity of central pathways of the reflex. Same animal as in Fig. 2.

as mediated by pressure-induced activation of aortic nerve C f bres—and that the C fibres had probably contributed to the reflex response shown in Fig. 3 d, with the aortic nerve intact.

The higher threshold for activation of receptors with C than A fibres was confirmed in all rabbits (Fig. 4). A 20–30 mmHg rise in pressure, corresponding to the threshold of C fibre reflexes, caused a 60% decrease in sympathetic discharge when receptors with A fibres were allowed to participate. Related to maximum mean pressure (Fig. 4 right), aortic nerve C fibres contributed to the sympathetic inhibition only at pressures exceeding 110 mmHg, whereas with A fibres operative, the inhibition was almost complete from 100 mmHg upwards.

I an attempt to assess the firing frequency of aortic C fibres during the reflex events depicted in Fig. 4, we recorded the sympathetic responses to electrical, combined stimulation of A and C fibres in the left aortic nerve (Fig. 5). The stimulation was usually performed ithout simultaneous occlusion of the left carotid artery and in a frequency range where selectiv activation of A fibres is known to be devoid of reflex effects (Douglas et al. 1956). This as tested and confirmed in present rabbits. Accordingly although the results plotted in Fig. 5 ere obtained by combined A and C f bre stimulation, they represent reflex effects of activity in C fibres alone. Pulses of 4 Hz were found to produce a 65% reduction in sympathetic discharge, while ma imum effects were obtained by about 10 Hz. These responses were not altered by concomitant carotid occlusion or anodal block of the A fibres. ectrical stimulation or pressure increases produced only negligible changes in heart rate. lon of noradrenaline (5 μg/kg min) initially led to a rise in pressure and inhibition of

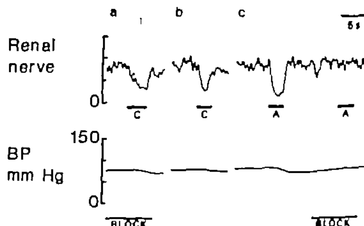


Fig. — Specificity of the anodal block of A fibres in left aortic nerve demonstrated by recordings of reflex changes in average renal nerve activity. Stimulation of aortic nerve distal to blocking site, with pulses 1.5 ms, 6 V 20 Hz (marked C) and 0.02 ms, 6 V 100 Hz (marked A). C fibre stimulation depressed nerve activity to the same level with as without anodal block, while the effect of A fibre stimulation was abolished by the block.

An ideal block usually obtained with 5–15 μA, was characterized by complete and immediate block of conduction in A fibres and no inhibitory or excitatory effects on the C fibres. Higher currents blocked both types of fibres. The block was frequently controlled, and when necessary adjusted, by studying the evoked compound potentials in the aortic nerve (Fig. 1) and the reflex changes in the renal nerve activity (Fig. resulting from stimulation of the aortic nerve. The nerve was stimulated with pulses of 0.02 ms, 6 V 100 Hz, or 1.5 ms, 6 V 0.5–20 Hz, for excitation of A fibres or A and C fibres combined, respectively. Recorded aortic nerve activity was displayed on an oscilloscope and monitored with a loudspeaker.

The actual experiment consisted of recording the peak sympathetic responses to a 5–10 s rise in arterial pressure in two different situations: with the left aortic nerve intact, and with conduction through the A fibres blocked. Reflex effects via baroreceptors of the left carotid sinus were avoided by transitory occlusion of the left common carotid artery during the pressure rise. The right carotid artery was permanently occluded by the arterial catheter and the right aortic nerve was cut. Duration of the blocking periods never exceeded 20 s. As conduction in the A fibres recovered immediately or within a few seconds after cessation of the block, several series of these experiments, with different increases in pressure, could be performed in each animal. In some rabbits, attempts were made to repeat the procedures during i.v. infusion of adrenaline. Blood pressure was then maintained close to control levels by bleeding the animals. At the end of all experiments, we recorded the sympathetic response, if any, to a pressure rise during carotid occlusion after the left aortic nerve had been cut.

These studies were made in a considerable number of rabbits. Results presented in this paper are from 12 rabbits fulfilling the following criteria: resting mean arterial pressure between 70 and 109 mmHg, the anodal block frequently controlled, and no alterations throughout the experiment in sympathetic response to aortic nerve stimulation or a rise in pressure.

Results

Typically a 30–35 mmHg rise in mean arterial pressure caused severe inhibition of sympathetic discharge when aortic A and C fibres were intact, but a barely noticeable drop when the A fibres were blocked (Fig. 3 b, c). Sympathetic inhibition occurred, however, when during anodal block of A fibres the pressure was increased by about 50 mmHg (Fig. 3 The immediately preceding demonstration of selective block of the A fibres (Fig. and the subsequent diminutive response to the same pressure rise after the left aortic nerve had been cut (Fig. 3 f), indicated that the reflex sympathetic depression shown

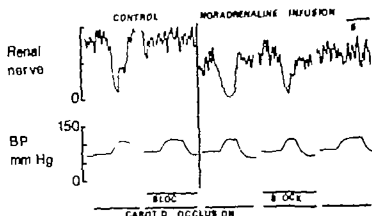


Fig. 6 Effect of noradrenaline (5 µg/kg min) on threshold for excitation of baroreflex via aortic nerve C fibres. Procedures as in Fig. 3. Note reduced sympathetic activity despite equal pressure (obtained by bleeding) during infusion of noradrenaline, and reflex sympathetic inhibition by a pressure rise which prior to the infusion had no effect in C fibres. The last recording shows the lack of effect after the left aortic nerve had been cut

Discussion

Our results demonstrated that a larger rise of pressure was needed to elicit reflex sympathetic inhibition via aortic nerve C than A fibres. Most likely this means that receptors with C fibres have higher thresholds than receptors with A fibres. At least in theory however, receptor threshold and central nervous responses to C fibre activity might have been modified by the block of efferent or afferent traffic in the A fibres, by carotid occlusion, or by the anesthesia. Efferent stimulation of the aortic or sinus nerves has been shown not to affect baroreceptor activity in these nerves, but the investigation was probably limited to receptors with A fibres (Neil and O'Reagan 1971). As for influences on central pathways of the C fibres by cessation of afferent activity in baroreceptor A fibres, our findings of equal responses to stimulation of C fibres before, as during carotid occlusion or anodal block suggest that such interactions were not of importance. The carotid occlusion might have activated carotid chemoreceptors, thereby interfering with the baroreflex (Korner et al. 1973, Ott and Shepherd 1973, Pelletier and Shepherd 1975). However, the occlusion was probably too brief for this to happen (Pelletier and Shepherd 1975), since neither sympathetic activity nor blood pressure showed signs of secondary increases during the occlusion, and—as just mentioned—responses to stimulation of the aortic nerve remained unaffected. A number of anesthetic agents are known to increase baroreceptor sensitivity, but the studies have all been limited to A fibres (Price and Widdicombe 1962, Biscoe and Millar 1964, Hagenau, Pietsch and Arndt 1976). Different effects on A and C fibre receptors, like that described for pulmonary high- and low-threshold stretch receptors (Coleridge et al. 1968), cannot be totally excluded, the same applies to the central pathways of the two types of fibres (Biscoe and Millar 1966). On the whole, however, we believe that the experimental procedures did not unduly influence our results.

... vascular resistance in cats is known to change in parallel with systemic arterial

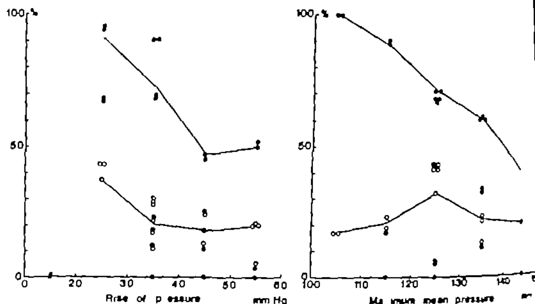


Fig. 4 Reflex reductions in renal sympathetic discharge mediated by aortic nerve C f bres (filled symbols) or A and C f bres (pen symbols) in response to a 5–10 s rise in mean arterial pressure. *Left* Related to magnitude of pressure rise. *Right* Related to maximum pressure reached by the rise. Resting pressure levels 70–109 mmHg. 84 observations from 10 rabbits each nimal represented by its mean value of all observations for each 10 mmHg step Lines re medians.

sympathetic activity The nerve activity remained lower than normal when, after a few minutes, blood pressure had been adjusted to pre infusion levels. Rises of pressure too small to inhibit sympathetic discharge through activation of C fibres *before* the infusion now caused a marked sympathetic depression (Fig. 6)

Sectioning of the left aortic nerve usually abolished the sympathetic response to a rise of pressure during carotid occlusion (Fig. 6) A small sympathetic reduction was occasionally seen (Fig 3 f), but this developed more slowly than before, and disappeared after the vagus nerves had been cut

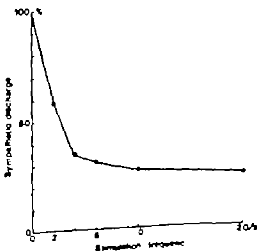


Fig. 5 Renal nerve responses to electrical stimulation of A nd C f bres in the whole left ortic nerve (ms, 6 V in periods of 5). Control mean pressures 70–109 mmHg. Left carotid sinus f fluences from right aortic nd al us nerves cluded. The media of bservations from 10 animals with 95 confidence intervals shown by shaded

ed ith Nembutal, a 20–30 mmHg increase in systolic pressure was found to be insuf ent for elicitation of reflex bradycardia via aortic nerve C f bres (Aars and Myhre 1974). dged by the present results, this was to be expected, since the pressure stimulus must have en too low to trigger activity in the C fibre receptors.

The extreme sensitivity of sympathetic neurons to electrical stimulation of aortic nerve fibres (Fig. 5) agrees well with observations by other authors. Thus, Ninomiya *et al* (1971) und that bilateral stimulation of aortic nerve C f bres in cats at 2 impulses/heart beat qual to about 4 Hz) caused a 50 % reduction in average renal nerve activity 8–16 s after art of stimulation. A similar effect was observed in sympathetic fibres to skeletal muscle pon stimulation of the vagodepressor nerve, also in cats (Koizumi and Sato 1972). Blood ressure responses to stimulation of C fibres in the rabbit's aortic nerve reached 60% of aximum at 4 Hz (Douglas *et al.* 1956). That is almost identical to the effect of the same imulation on activity in the renal nerve (Fig. 5)

A strict comparison between sympathetic responses to pressure-induced and electrically voked activity in aortic nerve C fibres is probably not warranted, since the aortic nerve ay contain fibres from receptors outside the aortic arch, *e g* from the heart (Sarkar 1922, Douglas and Ritchie 1956). Also the pattern and central impact of individual discharge nduced by the pressure rise most likely differed from that evoked by electrical stimulation f all C fibres in the nerve. With due respect to these reservations, however our results ould seem to suggest that, on the whole, *i*, C fibres of the rabbit's aortic nerve have no or negligible spontaneous activity at normal arterial pressures, *ii*, their receptors have thresholds t about 30 mmHg for abrupt rises in mean pressure and above 110 mmHg for steady mean pressure, and *iii*, their a erage discharge rate is low probably less than 5 imp/s for mean pressures up to 150 mmHg. Landgren (1952), who was the first to study in detail the characteristics of thin and thick baroreceptor fibres, found that the "small spike fibres the cat sinus nerve had thresholds for steady pressures at 120–150 mmHg, as compared to 80–120 mmHg for the "large spike fibres Very likely these were non-myelinated and myelinated fibres, respectively The difference in threshold between the two has later been suggested or confirmed by others (Fidone and Sato 1969 Coleridge *et al.* 1973 a) Thorén, Saum and Brown (1977 a) presented the first study of individual baroreceptor C fibres, in hich the fibres were grouped according to conduction velocities and type of firing. Utilizing controlled perfusion of the aortic arch, they found that, when tested by a pressure ramp, the threshold for the most commonly occurring type of C f bres in the rat's aortic nerve was 152 mmHg (range 85–260) From our deductions, w would expect about the same to be found in the rabbit

In the one rabbit successfully studied, noradrenaline induced a pressure-independent depression of sympathetic activity and a reduction in pressure threshold for receptors with aortic nerve C fibres. These findings support the conclusions of previous studies in this laboratory (Akre and Aars 1977), in suggesting that the C fibre receptors constitute the target and the means of efferent sympathetic control of the baroreflex. Similarly Thorén *et al* (1977) observed that addition of noradrenaline to the aortic arch perfusate led to lowering of the threshold of individual aortic nerve C fibres. Unfortunately most of our ts at studying the effect of noradrenaline on the reflex failed, due to deterioration of

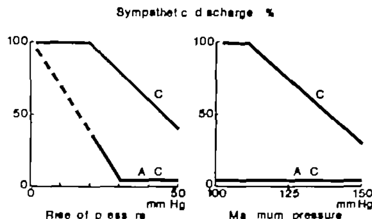


Fig. 7 Participation of aortic nerve A and C fibres in the baroreflex control of renal nerve activity, as elicited by a 5–10 s abrupt rise in mean aortic pressure. Schematic presentation based on Fig. 4 after subtraction of pressure-independent activity

pressure and vascular resistance in skeletal muscle during elevation of carotid sinus pressure up to 200 mmHg (Kendrick, Öberg and Wennergren 1972, Little, Wennergren and Öberg 1975) Whether this balance is limited to the activation of A fibres with different receptor thresholds, or if it holds for baroreceptor C fibres as well is presently largely unknown. Ninomiya *et al* (1971) found that the stimulation of aortic nerve C fibres in cats reduced sympathetic activity to the spleen and the kidney to about the same degree. In rabbits, we have often observed a closer correlation between the reductions in renal nerve activity and blood pressure during stimulation of aortic nerve C than A fibres. We certainly have never seen indications of an arrangement of the sympathetic supply to the kidney similar to that of the cardiac sympathetic neurons, which have been found to be without projections from baroreceptor C fibres (Kardon *et al* 1975)

In order to arrive at a simpler presentation of the results plotted in Fig. 4 we have in Fig subtracted the baroreceptor independent activity which remained during maximum responses to A and C fibre activity (Fig. 4 and 5) and straightened the lines slightly Fig then presents, in a schematic way the contribution of aortic nerve A and C fibres to the baro reflex control of sympathetic vasomotor neurons projecting to the kidney Assuming—as just discussed—that changes in renal sympathetic activity are fairly typical of the baroreflex Fig. 7 would apply to the control of vasomotor activity also to other vascular beds engaged in the baroreflex. It is important to realize that this illustration of the reflex is valid only for peak sympathetic responses to a pressure step The reflex effects undoubtedly depend upon the rate of rise of pressure but this unfortunately could not be systematically studied with the present technique. Instead we tried to keep the rate of rise constant. This often proved impossible during infusion of noradrenaline, when, due to volume depletion and reduced cardiac output, the rate of pressure rise became very low High steady pressures, which would have been better suited for determination of thresholds, were difficult to obtain especially in combination with the desired steep rising phase Also occlusion of the descending thoracic aorta in rabbits after about 30 s is apt to induce a secondary marked increase in efferent renal nerve activity (Aars and Akre, unpublished results)

Influence on the heart rate of pressure-induced activity in A and C fibres, respectively could not be determined in the present expts., due to the vagolytic effect of chloral and urethan (Korner Uther and White 1968) However in our earlier studies in rabbi

ed with Nembutal, a 20–30 mmHg increase in systolic pressure was found to be insuf ient for elicitation of reflex bradycardia via aortic nerve C fibres (Aars and Myhre 1974). idged by the present results, this was to be expected, since the pressure stimulus must have een too low to trigger activity in the C fibre receptors.

The extreme sensitivity of sympathetic neurons to electrical stimulation of aortic nerve fibres (Fig. 5) agrees well with observations by other authors. Thus, Ninomiya *et al.* (1971) ound that bilateral stimulation of aortic nerve C fibres in cats at 2 impulses/heart beat equal to about 4 Hz) caused a 50 % reduction in average renal nerve activity 8–16 s after tart of stimulation. A similar effect was observed in sympathetic fibres to skeletal muscle upon stimulation of the vagodepressor nerve, also in cats (Koizumi and Sato 1972). Blood ressure responses to stimulation of C fibres in the rabbit's aortic nerve reached 60 % of maximum at 4 Hz (Douglas *et al.* 1956). That is almost identical to the effect of the same timulation on activity in the renal nerve (Fig. 5).

A strict comparison between sympathetic responses to pressure-induced and electrically evoked activity in aortic nerve C fibres is probably not warranted, since the aortic nerve may contain fibres from receptors outside the aortic arch, *e.g.* from the heart (Sarkar 1922, Douglas and Ritchie 1956). Also the pattern and central impact of individual discharge nduced by the pressure rise most likely differed from that evoked by electrical stimulation of all C fibres in the nerve. With due respect to these reservations, however our results ould seem to suggest that, on the whole, *i*, C fibres of the rabbit's aortic nerve have no or negligible spontaneous activity at normal arterial pressures, *ii*, their receptors have thresholds at about 30 mmHg for abrupt rises in mean pressure and above 110 mmHg for steady mean pressure, and *iii*, their average discharge rate is low probably less than 5 imp/s for mean pressures up to 150 mmHg. Landgren (1952), who was the first to study in detail the characteristics of thin and thick baroreceptor fibres, found that the small spike fibres in the cat sinus nerve had thresholds for steady pressures at 120–150 mmHg, as compared to 80–120 mmHg for the "large spike fibres. Very likely these were non-myelinated and myelinated fibres, respectively The difference in threshold between the two has later been suggested or confirmed by others (Fidone and Sato 1969 Coleridge *et al.* 1973 a). Thorén, Saum and Brown (1977 a) presented the first study of individual baroreceptor C fibres, in hich the f bres ere grouped according to conduction velocities and type of firing. Utilizing controlled perfusion of the aortic arch, they found that, when tested by a pressure ramp the threshold for the most commonly occurring type of C fibres in the rat's aortic nerve was 152 mmHg (range 85–260). From our deductions, we would expect about the same to be found in the rabbit.

In the one rabbit successfully studied, noradrenaline induced a pressure-independent de pression of sympathetic activity and a reduction in a pressure threshold for receptors with aortic nerve C fibres These findings support the conclusions of previous studies in this laboratory (Akre and Aars 1977), in suggesting that the C fibre receptors constitute the target and the means of efferent sympathetic control of the baroreflex. Similarly Thorén *et al.* (1977 a) observed that addition of noradrenaline to the aortic arch perfusate led to lowering of the threshold of individual aortic nerve C fibres. Unfortunately most of our ts at studying the effect of noradrenaline on the reflex failed, due to deterioration of

the preparations late in the day and difficulties in producing pressure rises similar to the obtained prior to infusion of noradrenaline.

Complete functional separation of A and C fibres in the aortic nerve was crucial to t present experiments. Judged by the effects of the block on evoked action potentials (Fig and on sympathetic responses to aortic nerve stimulation (Fig. 2), such a separation reached in all experiments presented in this paper Our main difficulties with the block w electrical damage to the nerve and as reported by others (Coleridge *et al* 1973 b, San and Zimmermann 1973 Fukushima, Yahara and Kato 1975 Thorén, Shepherd and Don 1977) excitation of C fibres These complications were virtually abolished by improve of electronic equipment in particular by avoidance of electrical noise in the blocking circ The slow rise of the current as well as the much lower currents required for anodal bl of A fibres in the aortic than *e g* the vagal nerves (Trenchard 1970, Sant Ambrogio *et* 1972, Coleridge *et al* 1973 b Thorén *et al* 1977 b Kardon *et al* 1975) were probably of importance. The occurrence of a frequency-dependent block of C fibres, shown to during anodal block of vagal A fibres in cats (Thorén *et al* 1977 b) was assured against accepting only anodal blocks which did not affect the sympathetic response to C fi stimulation at 10–20 Hz frequencies well above the expected spontaneous discharge in the C fibres Failure to meet this requirement led to rejection of many expts. Howev with experience, the anodal block in our hands proved a useful and safe, although ti consuming tool which enabled us to separate, for the first time, the contribution of ao nerve A and C fibres to the pressure induced baroreflex inhibition of sympathetic ac activity

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N te added in proof *A study of the characteristics of aortic baroreceptor C f bres in the rabbit*, based single f bre recordings, w recently publ shed by Thorén and Jones (*Acta physiol scand.* 1977 *99* 448-

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Tissue osmolality in intestinal villi during luminal perfusion with isotonic electrolyte solutions

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Abstract

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A cryoscopic technique has been developed that makes it possible to determine tissue osmolality in t[illegible] core of the intestinal villi. During [illegible]bsorption from an isotonic electrolyte solution containing gluco[illegible] osmolality gradient was demonstrated from tip to base of the villi in both the jejunum and the ileum T[illegible] tissue osmolality at the villous tips was measured t[illegible] 1 000–1 200 mOsm/kg H_2O while the osmolality [illegible] the villous bases was approximately isotonic with plasma. Increasing intestinal blood flow by [illegible] administration of a vasodilator drug, or making the intestine ischemic by clamp[illegible]g the intestinal vascu[illegible] supply while supplying the mucosa w[illegible]th [illegible]xygen, markedly decreased tissue osmolality Substituting [illegible] sodium ions with choline in the luminal perfusat[illegible] [illegible]bolished almost completely the tissue hyperosmola[illegible] and the intestine became a secretory organ. These observations are consistent with the [illegible]iew that the observ[illegible] villous tissue hyperosmolality was created by a countercurrent multiplication of sodium chloride T[illegible] physiological implications of this mechanism is discussed and it is, among other things, proposed that t[illegible] hyperosmolar region represents the hyperosmotic compartment necessary for explaining intestinal wat[illegible] absorption

The vascular arrangement of intestinal villi is consistent with the existence of a counte[illegible] current exchanger (for reviews, see Lundgren 1967 1974) In the cat the exchanger [illegible] formed by an arterial vessel running in the central part of the villous core and by [illegible] extensive network of subepithelial capillary vessels surrounding the artery It was propo[illegible] by Jodal and coworkers (Haljamäe *et al* 1973 Jodal 1973) that the most importa[illegible] physiological implication of the exchanger was its significance for water and sodium absor[illegible] tion by creating the hyperosmolar compartment necessary to explain water absorption [illegible] the absence of or against, a lumen to plasma osmolality difference (see Curran and McInto[illegible] 1962, Schultz and Curran 1968). This is accomplished if the exchanger acts as a counte[illegible] current multiplier in a similar way as in the kidney papillae

In the cited study by Haljamäe *et al* (1973) evidence for the countercurrent hypothesis was obtained by determining the sodium concentrati[illegible] d a

tissue sodium per unit weight of tissue protein, along the length of the villus. These dies revealed that the villous tip contained 3–4 times as much sodium per unit weight otein as the villous base implying that a considerable hyperosmolality may exist at the portion of the villus.

The present study was carried out to obtain more direct evidence for the hypothesis oposed by Jodal *et al.* It describes a cryoscopic technique that makes it possible to deter mine tissue osmolality along the intestinal villi of the feline gut (length 0.7 1 mm). The sults obtained with this technique, described below provide strong support for the untercurrent multiplication hypothesis.

Methods

Operative procedures

e experiments were performed on cats anesthetized ith chloralose (50 mg/kg b t) after duction ith ether. The animals had been deprived of food for 24 h and had no obvious signs of intestinal ection. The operative procedures as well as the recordings of blood flow and of net water bsorption re similar to those described by Jodal *et al.* (1975). Briefly total venous outflow from one or two mpathetically denervated intestinal segments, either from mid-jejunum and/or from ileum, as recorded drop recorder unit operating an ordinate riter. Mean arterial blood pressure was measured from left femoral artery by pressure transducer (Statham P23AC). Cholinergic influences were eliminated atropine administration, 1 mg/kg b wt. I some experiments intestinal vasodilatation was achieved close infusion of isopropylnoradrenaline (20 µg/ml) at rate of 5–10 µg/min ere cannula in all branch of the superior mesenteric artery.

In one series of experiments, where net water bsorption was not monitored (see below), small jejunal gments were opened along the antimesenteric border by thermocautery and mounted on metal frames described by Haljamäe *et al.* (1973). By means of plastic frame small bath was then created over the posed mucosa. The bath was flushed at constant rate of about 1 ml/min. The temperature of the lution was controlled by thermocouple thermometer (Electrolab, Copenhagen) and kept at 38°C by ating pad. Initially the mucosa as flushed for 30 min with choline-mannitol solution (see below). The testinal segment was then made ischemic by clamping its vascular supply and the solution flushing the ucosa as simultaneously changed to Krebs-glucose solution (see below). In this situation oxygen was livered to the tissue via the mucosal solution by bubbling the solution with oxygen.

Recording of intestinal net water transport

testinal net water transport as continuously measured with recirculating system that was coupled the lumen of the intestinal segments and contained large reservoir (volume 1 000 ml) to minimize circulation. The olume change of this system was recorded with volume transducer connected via tube to the perfusion system. Provided no motility occurred the recorded change of volume reflected et water bsorption or secretion (see Jodal *et al.* 1975). The intraluminal pressure of the outflow end of e segment as kept at 1 cm H_2O and the intestinal segment as perfused at constant rate of about ml min by means of roller pump (Model mp-4, Ismatec Sa, Zurich, Switzerland). The temperature f the perfusate entering the segment was continuously monitored with thermocouple thermometer Electrolab, Copenhagen) and kept at 38°C by heating pad.

Cryoscopic technique

issue cryoscopy has earlier been used particularly in the kidney. The reports by Wirz *et al.* (1951) and mery *et al.* (1972) have been of great help in developing the present method for the gut.

General procedures. After at least 20–25 min of constant net water transport or after 45 min incubation f the "opened" intestines, the segments were rapidly extirpated and momentarily frozen in isopentane, recooled to ~160°C in liquid nitrogen. The intestinal wall as then cut obliquely in cryostate kept at 20 to 25°C so as to gi cross-sections at different levels of the villi. The tissue sections (10–15 µm re embedded in kerosene between glasses (20 30 0.8 mm) and transferred to temperature unit placed in room kept at 10 C. This unit consisted of an aluminum block (100 30

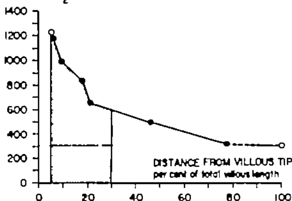


Fig. 1 The computer treatment of the osmol. measurements made on an intestinal tissue tion The actual observations are indicated filled circles. The line connecting the two upm ings of tissue osmolality as extrapolated to t 5 % level of villous length (unfilled circle). Fu thermore the osmolality at the base (unfil. circle) was assumed to be that of plasma (ie 3 mOsm/kg H O also indicated by the h horizontal, dotted line). Shaded part of the Fi denotes the area under the curve from 5 to 3 of total villous length, which is used t estimate mean osmolality in this part of t villus (see Table 1)

15 mm) furnished with a hole (diameter 8 mm) into which a plastic cylinder was tightly fitted ith silic grease The luminium block was insulate with Frigol te® 5 mm thick except for a rectangular area ov the hole into which the slides f tted The temperature of the aluminl m block was controlled by a sys system nd could be set at any temperature between −4.5 and +0 5°C. The whole system as placed the objective table of a Zeiss standard microscope so that a y part of the transluminated tissue sect could be focused and photographed in the microscope A thin layer of silicone-oil was placed between t aluml um block nd the slides to assure good contact and thus a homogeneous and constant temperat. of the tissue section Thawing of the i testinal tissue was achieved by increasing steps the tissue te perature, usually in 3–6 steps with at least a 10 m equilibration period between each step. The amou of tissue that was thawed t each temperature was then determined from photographs taken at the e of the equilibratio period as will be described below

2. *Calibration* The temperature f the l minium block could be adjusted between +0 5 to 451 with a potentiometer and the readings of this was linearly related to the temperature of the alumin block Since tissue temperat re could not be assumed to be identical to that of the metal blocks, the m scopic system was calibrated This was done in two principally d fferent ways. First, the melting poin sal ne solutions of d fferent osmolalities were determined when the solutions were frozen either as t layers betwen cover glasses t −10°C o as cylindrical blocks (l me 1 ml) i Isopentane precool with liquid nitrogen. I the latter case albumi was added to the solution to facilitate the cutting of the in 15 μm thick sections. Second, small everted testinal rings from u cats were incubated for 60 m Krebs solutions in which the osmolalities were varied betwee 300 nd 1 000 mOsm/kg H_2O by adding appropriate mounts of mannitol. To void changes i osmolality of the incubatio medium, lar volumes (round 700 ml) were used in each bath. The baths were bubbled with gas containing 5 C in O_2 and the temperature was kept t 21 23°C. After the incubation period the intestinal segments w quickly frozen in liquid nitrogen and cut t 20 to −25 C in 10 μm thick sections. The thi ice or sections were then treated exactly as the testinal segments (see below)

3. *Determination of tissue osmolalu* The temperat re at which the last ice crystal thawed in a vill cross-section was taken as the tissue melt ng poi t. The microscopic magnifications used (17 or 7 were too low to allow any observation of tracellula ice crystals i the villous tissue. Thus, the me melting point determined i this study is considered t be a measure of the extracell lar osmolality

The thawing of the intestines, btained during *in vivo* conditions, alway tarted t the llous tips gradully spred downwards until, f nally the crypt region, the submucosa and the muscularis melted largely the same temperature (see Result). The percentage of llous tissue melted t each temperatu was determined from the photographs and plotted llustrated n Fig 1 Usually 3–6 readings w btained on each section, the tip alue normally fall g within the tip 5–15 per cent of the villous leng The data were treated in a computer i the way indicated in Fig. 1 / the line connect g the two t values was extrapolated to the 5 per cent level. Furthermore the base osmolal ty was ssumed to be eq t that of plasma (around 312 mOsm/kg H_2O). From this curve the computer calculated the tiss osmolality at 5 10, 20, 30, 40, 50, 60, 70, 80 and 90 per cent of villous length as the mea

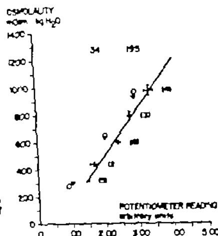


Fig. 2. Calibration curves for the cryoscopic method. The potentiometer reading is directly proportional to the temperature of the aluminium block. In one type of expts. the calibration was performed using sodium chloride solutions of varying osmolalities with (open circles) and without (triangles) albumin. Each point represents mean value of 2–4 observations. In another type of experiments the calibration was performed using intestinal tissue sections exposed to Krebs-mannitol solutions of different osmolalities (dots). The line drawn and the equation given in the Fig. refer to these expts. The line was drawn according to the method of least squares. Figures in brackets denote number of observations. Bars indicate ± E.

between 5 and 30, 5 and 50, and 5 and 100 per cent villous length. There are two reasons for not including the tip 5 per cent in the calculations of mean tissue osmolality. First, a large portion of the tip consists of epithelial cells, the osmolality of which probably is lower than that of the villous core (see Discussion). Second, the villus is not a true cylinder but tapers off at the tip.

In the calibration experiments using intestinal segments, the whole intestinal tissue reached at the same temperature. The tissue osmolality was assumed to be that of the incubation medium.

Solutions

The intestinal mucosa was exposed to the following solutions:

Krebs-glucose solution. This modified Krebs-Henseleit solution contained (mmol/l, pro analysi): NaCl 122, KCl 4.7, $NaHCO_3$ 21, KH_2PO_4 1.2, $MgCl_2 \cdot 6H_2O$ 1.2, $CaCl_2$ 2.5, glucose 30.

Krebs-mannitol solution. This solution was the same as the Krebs-glucose solution except that glucose was exchanged for an equal amount of mannitol.

Choline-mannitol solution. This solution was the same as the Krebs-mannitol solution except that all sodium was substituted with choline.

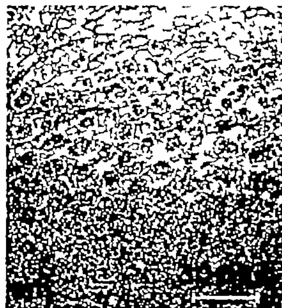
The osmolality of the three solutions ranged between 305 and 315 mOsm/kg H_2O. If necessary, the osmolality of the solutions was adjusted to a value within 10 mOsm/kg H_2O of that of plasma by addition of appropriate amounts of mannitol.

Statistics

Statistical significance was tested using Wilcoxon's nonparametric tests. A p-value of 0.05 or less was judged as significant.

Results

With both methods of calibrating the cryoscopic system a linear relationship was demonstrated between temperature of the aluminium block and osmolality as illustrated in Fig. 2. The results obtained with the two saline solutions were similar independent of the method of freezing used. On the other hand, the calibration curve obtained with tissues of different osmolalities was clearly shifted to the right, *i.e.* the same temperature of the aluminium block represented a lower osmolality compared to the first mentioned method. As the tissue method was considered to be the most correct one, the tissue osmolality values given below are determined with this calibration curve.


a

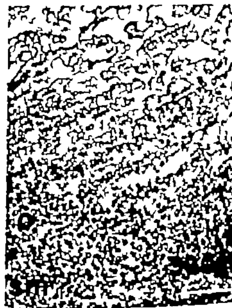
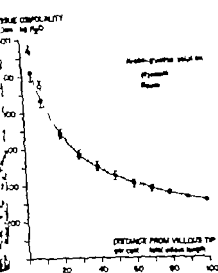

b

Fig. 3 *Left panel* Tissue section from a jejunal segment exposed to an isotonic Krebs-mannitol sol photographed at a temperat re corresponding to freezing poi t depression caused by solution an osmolality of 1 160 mOsm/kg H_2O The crypt region (c) and the submucosa (sm) are frozen while villous crossections close to the intestinal lumen (upper part of the Fig) are completely thawed. *Right* The same tissue section photographed t a temperature corresponding to a freezing poi t depr caused by a solution of 660 mOsm/kg H O

The thawing of the intestinal wall always started at the villous tip and proceeded tow the base as the tissue temperature was stepwise increased This is illustrated in Fig. 3 w is taken from an experiment where the lumen was perfused with a Krebs-mannitol solu In the left panel of Fig. 3 the temperature of the tissue corresponds to a depression o freezing point that is caused by a solution with an osmolality of 1 160 mOsm/kg H_2 is clearly seen that only cross-sections at the very tips of the villi are thawed, whil submucosa, the crypt region and the basal half of the villi are still frozen. Ice crystal also observed in the intestinal lumen surrounding the thawed cross sections. In the bo line zone between melted and completely frozen tissue the ice crystals in the cross-sec are enlarged and reduced in number (Fig. 3)

A change of tissue temperature to a value equivalent to an osmolality of 660 mOs H_2O resulted in a further thawing of the villi as showed in the right panel of Fig. 3 He crystals are observed only in the basal parts of the villus, in the crypt region and in the mucosa-muscularis. The ice crystals in the intestinal lumen adjacent to the villous c sections appear to melt at a somewhat higher temperature than the corresponding c section. When tissue temperature was increased to or above that corresponding to a fre point depression caused by 310 mOsm/kg H_2O the whole tissue section includin submucosa and the muscularis, melted

Thus, the results of Fig. 3 strongly suggest that there exists a tissue region at the vi tip which has a considerably higher osmolality than that of the fluid perfusing the



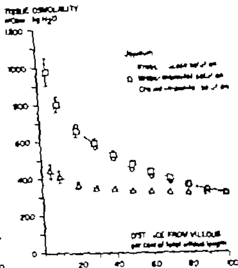


Fig. 4

Fig. 5

Fig. 4. Tissue osmolality in villi from jejunal (n 14) and ileal (n 14) segments during absorption from isotonic Krebs-glucose solution during resting blood flow. Bars denote S.E.

Fig. 5. Tissue osmolality in villi of the jejunum when perfusing the gut lumen with three different isotonic solutions. Intestinal blood flow was within the resting range. When exposed to the choline-mannitol solution the jejunal segment secreted fluid into the lumen. Bars denote S.E. Number of observations, see Table I.

men or that of plasma. Moreover there exists an osmolality gradient within the villus from tip to its base.

Fig. 4 illustrates the compiled data for the osmotic gradient along the villus in both jejunum and ileum when the lumen was perfused by an isotonic Krebs-glucose solution. The tip osmolality was estimated to 1 139 ± 31 mOsm/kg H_2O ($x \pm$ S.E., n 14) in the jejunum and to 1 026 ± 47 mOsm/kg H_2O (n 14) in the ileum. These values differ significantly from each other while no significant difference could be demonstrated for the rest of the values illustrated in Fig. 4. Mean osmolality values for different villous portions in the jejunum and the ileum (see Methods) are given in Table I.

The efficiency of a sodium countercurrent multiplier depends, among other things, on the rate of active sodium transport and the magnitude of certain circulatory parameters in the villi. Fig. 5 and 6 illustrate the effects of reducing the rate of sodium absorption on the villous osmolality gradient in jejunum and ileum. The decrease in sodium absorption was accomplished in two ways, i.e. either by exchanging glucose with mannitol in the normal perfusate or by replacing all the sodium in the perfusate with choline. The elimination of glucose in the perfusate reduced the villous tip osmolality to 975 ± 72 mOsm/kg H_2O (n 10) in the jejunum and to 979 ± 70 mOsm/kg H_2O (n 9) in ileum, the reduction being statistically significant in the jejunum but not in the ileum. Exposing the intestinal mucosa to an isotonic solution containing no sodium ions (choline-mannitol) reduced the villous tip osmolality drastically to 442 ± 39 and 391 ± 24 mOsm/kg H_2O in the jejunum

TABLE I Mean tissue osmolality in mOsm/kg H O in various portions of intestinal vill during different experimental situations of this study Mean values ± S.E.

	Number of observations	From 5 to 30 per cent of villous length	From 5 to 50 per cent of villous length	Whole villus
Jejunum				
Krebs-glucose resting blood flow	14	774±23	654±20	540±13
Krebs-glucose, vasodilatation	1	570± 1	514±21	473 [illegible]
Krebs-glucose, ischemia	19	471±11	440±11	[illegible]
Krebs-mannitol	10	711±35	629±27	550±19
Choline-mannitol	10	378± 4	360±18	344 [illegible]
Ileum				
Krebs-glucose	14	743±32	634± 4	530±16
Krebs-mannitol	9	769±48	674±37	569±[illegible]
Choline mannitol	11	351±16	343±13	337±11

and the ileum, respectively In this situation the intestinal segments become secre organs.

The villous circulation of blood was also altered in two ways. In one series of experim the total venous outflow from the intestinal segments was increased from a resting val of 28±2 ml (min 100 g) (n = 7) to 147±16 ml (min 100 g) by means of close i infusions of isopropyl noradrenaline. In this situation the mean transit time for plasma thro the villous vascular loops simultaneously decreases from 4–6 sec at "rest" to 1 sec or l during vasodilatation (Biber *et al* 1973) Concomitantly the hyperosmolality at the vill tip was reduced to 709±33 mOsm/kg H_2O (n = 12)

In another series of expts. the intestinal circulation was completely stopped by clamp the vascular supply Prior to the vascular occlusion these segments were exposed to choline-mannitol solution for 30 min During the ischemia the intestinal segment w exposed to a Krebs-glucose solution that was continuously equilibrated with a gas mixt containing 5% CO in O_2. Thus, the intestinal segments were in this situation incuba as during *in vitro* conditions. The tip osmolality value was reduced as compared both the values observed during "resting" and increased blood flow (Fig 7).

Table I summarizes the measured mean osmolalities in different portions of the villi the experimental series of Fig 5–7

Discussion

A prerequisite for determining osmolality in biological tissues by cryoscopy is a ra freezing of the tissue. The freezing process in a salt solution always starts with a crystal tion of water and a concentration of the remaining solutes until the entire solution solidified at the so called eutectic temperature. This temperature is characteristic for e solute and has for NaCl been determined to −21.8 C (Meryman 1966) A too slow free of the tissue may induce artefacts such as diffusion within an unfixed tissue and/or a extensive "salting out" phenomenon With the present freezing c hod emp

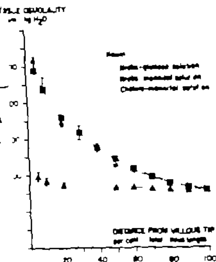


Fig. 6

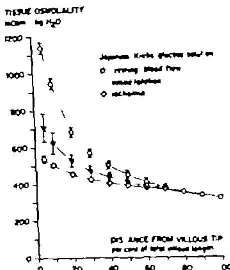


Fig. 7

Fig. 6 Osmolality in the villous tissue in ileal segments when exposing the intestinal lumen to 3 different isotonic solutions. Intestinal blood flow was within the "resting" range. During perfusion with the choline-mannitol solution the ileal segments secreted fluid. Bars indicate ± S.E. Number of observations, see Table I.

Fig. 7 Tissue osmolality in the villi of jejunal segments during three different circulatory conditions. Vasodilatations were induced by close i.a. infusions of isopropylnoradrenaline. Ischemia was produced by occluding the superior mesenteric artery. Bars indicate ± S.E. For number of observations, see Table I.

eutectic temperature for NaCl was probably reached within 2–4 s in the villi after the clamping of intestinal vessels, which strongly indicates that such artefacts were avoided in this study.

During thawing at temperatures above the eutectic temperature, concentrated salt solutions in liquid state will surround not yet melted ice crystals. When the frozen tissue is warmed slowly as in the present study diffusion of solutes and water may take place and reduce osmotic gradients. Hence, conclusions concerning osmotic differences within each villous cross-section were not considered possible. The values for tissue osmolality reported in this study are essentially representative for the villous core. The osmolality of epithelial cells was probably lower than that of the villous core but could not be determined with the method used.

The results of this study are in full agreement with the countercurrent multiplication hypothesis earlier proposed by Jodal *et al.* (Haljamäe *et al.* 1973, Jodal 1973, 1974). Thus, the presence of a marked hyperosmolar region was demonstrated in the upper parts of the villus reaching values above 1 000 mOsm/kg H_2O. Furthermore, an osmolality gradient existed within the villous tissue, the tip osmolality being highest. These results fully agree with the observations made by Haljamäe *et al.* (1973) when studying the sodium distribution in the villous tissue. Changing the rate of sodium absorption in the intestinal epithelium by addition or removal of luminal glucose in the jejunum produced the expected effects (Fig. 5 and 6), *i.e.* increases of sodium transport by adding glucose to the luminal perfusate

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and the ileum respectively In this situation the intestinal segments become water organs.

The villous circulation of blood was also altered in two ways. In one series of experiments the total venous outflow from the intestinal segments was increased from a resting value of 28±2 ml (min 100 g) (n = 7) to 147±16 ml (min 100 g) by means of close infusions of isopropyl noradrenaline In this situation the mean transit time for plasma through the villous vascular loops simultaneously decreases from 4–6 sec at "rest" to 1 sec or less during vasodilatation (Biber *et al* 1973) Concomitantly the hyperosmolality at the villus tip was reduced to 709±33 mOsm/kg H_2O (n = 12)

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Table I summarizes the measured mean osmolalities in different portions of the villus in the experimental series of Fig. 5–7

Discussion

A prerequisite for determining osmolality in biological tissues by cryoscopy is a rapid freezing of the tissue. The freezing process in a salt solution always starts with a crystallization of water and a concentration of the remaining solutes until the entire solution is solidified at the so called eutectic temperature. This temperature is characteristic for each solute and has for NaCl been determined to −21 8 C (Meryman 1966) A too slow freezing of the tissue may induce artefacts such as diffusion within an unfixed tissue and/or a too extensive "salting out" phenomenon With the present freezing method empl

[illegible] II

[illegible] common to all models in Fig. 8

1. Extracellular sodium concentration (Na_{EC}) and extracellular potassium concentration (K_{EC}) at villous [illegible] equalled 150 mmol/l and 4.0 mmol/l, respectively (Kjellmer 1965). K_{EC} at villous tip was set to 12 [illegible] and decreased along villous length in proportion to the measured osmolality.
2. The extracellular space equalled 22 [illegible] at the villous base (Esposito *et al.* 1972).
3. Extracellular sodium and chloride concentrations are equal.

[illegible] assumptions

Model A	*Model B*	*Model C*
Constant villous extracellular space equalling 22 per cent (Esposito *et al.* 1972). Sodium chloride contributed to extracellular osmolality throughout the villous to the same relative extent as at the villous base, where osmolality [illegible] set to 312 mOsm/kg H_2O. Activity coefficient for sodium chloride 0.92	1. See assumption 2 in model A. 2. Constant Na_{EC}/K_{EC} ratio along the villus. 3. Villous extracellular space calculated from other assumptions and varying between 22 and 30–35 per cent at base and tip, respectively	1. See assumption 1 in model A. 2. One-to-one exchange of sodium and potassium at baso-lateral cell membrane.

[illegible] models it is assumed that extracellular sodium contributes to extracellular osmolality to [illegible] same relative extent at the base and at the tip of the villus. Model B mimicks the situation [illegible] where water moves out of the cells, driven by the osmotic forces created by the counter[illegible] current multiplier. In model C the extravascular space was considered to be constant and [illegible] as further assumed that a one-to-one sodium-potassium exchange occurred at the cell-[illegible] membrane, since the NaK-ATP-ase driven sodium pump is supposed to function in this [illegible] way (see [illegible] Glynn and Carlish 1975; Rose 1976).

However, none of the proposed models is likely to represent the whole truth, since [illegible] countercurrent multiplication is such a complex mechanism that, for example, models B [illegible] and C may well exist simultaneously. Moreover, the diffusion of potassium and sodium [illegible] along their concentration gradients across the cell membrane has not been evaluated in [illegible] any of the models. It is therefore not surprising that certain "internal" inconsistencies exist [illegible] the models. For example, the intracellular osmolality seems to be higher than the [illegible] extracellular one particularly in model C. However, one tentative conclusion may be drawn [illegible] from all these models, namely that the intracellular sodium concentration in the epithelium [illegible] at the tip of the villus is high ([illegible] 100 mmol/l) which may be of great functional significance [illegible] for absorption of glucose and amino acids (see below). Such an increase of intracellular [illegible] sodium concentration is corroborated by some recent findings in the countercurrent system [illegible] of the rat renal papilla (Morgan 1977).

There are many functional implications of the intestinal countercurrent multiplier some [illegible] of which will be summarized here. The villous tips represent the parts of the villi that are in [illegible] the most "efficient" contact with the luminal contents. When perfusing the intestinal lumen [illegible] with an isotonic Krebs-glucose solution, the osmolality of the villous tips reached values [illegible] of about 1 100 mOsm/kg H_2O, creating a gradient of tissue to lumen osmolality of about [illegible] 800 mOsm/kg H_2O which represents an enormous pressure difference. If a solute can exert

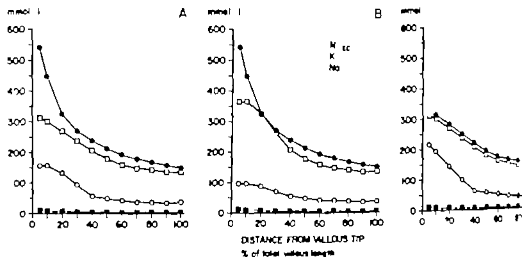


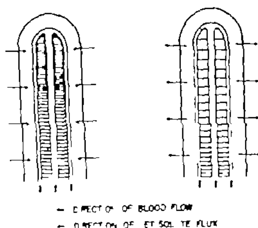
Fig. 8 Extracellular sodium concentration (Na_{EC}), extracellular potassium concentration (K_{EC}) ... cellular sodium concentration (Na_{IC}) and intracellular potassium concentration (K_{IC}) in the ... villi calculated from the results obtained in this study and the results reported by Haljamäe et al. (1973). The 3 panels are based on different assumptions described in detail in Table II

augments tissue osmolality particularly in the villous tips. Again, similar results ... obtained on the jejunum when investigating the distribution of sodium in the villi (Haljamäe et al 1973) Substituting all sodium ions in the luminal perfusate with choline abolished almost completely the tissue hyperosmolality (Fig. 5 and 6), strongly suggesting that ... countercurrent multiplication mainly involves sodium.

The efficiency of a countercurrent exchanger or multiplier is also dependent on cert... vascular parameters such as mean transit time in the villous vascular loops. This param... was altered in two ways. In one type of experiments vasodilatation was produced by ... propylnoradrenaline. This lowered villous osmolality markedly (Fig. 7) according to ... countercurrent hypothesis due to shortened mean transit time in the villi which reduces ... time for intervascular diffusion of electrolytes and water in the villous vascular lo... Similar results were obtained by Haljamäe et al (1973) when studying sodium distribut... It might be argued that isopropylnoradrenaline, a β-adrenergic agonist, may produc... effects at the cellular level However *in vitro* experiments suggest that isopropylnoradr... line has little or no effect on electrolyte transport in the intestine (Field and McCall 19...

In another type of expts. intestinal blood flow was completely stopped by occluding ... supplying vessels Tissue osmolality became decreased also in this experimental situa... as compared to the tissue osmolality gradient present during "resting" blood flow co... tions and during induced vasodilatation

Combining the present results with the values for tissue content of sodium and potas... reported by Haljamäe et al (1973) allows for an estimation of intra and extracel... sodium and potassium concentrations if certain assumptions are made Three such atte... have been pursued in Fig. 8 The various assumptions made in the different models ... summarized in Table II Briefly in model A the extracellular space has been consid... to be constant along the villus, while it in model B is supposed to vary keeping the i... cellular sodium and potassium concentration ratio constant. Furthermore, in these



9 Schematic illustration of proposed mechanisms by which papillary and villous structures may efficiently absorb or secrete fluid. In the left panel fluid absorption is accomplished by countercurrent multiplication of hypertonicity created by net solute flux into the subepithelial vessels. The rising tissue hypertonicity towards the tip of the papillary or villous structure is indicated by the increased number of horizontal lines. In the right panel secretion of fluid is produced by creating a hypotonic compartment (indicated by horizontal lines) at the tip by countercurrent multiplication of hypotonicity. This is created by net solute flux from the subepithelial capillaries into its surroundings. The mechanisms proposed are accompanied by osmolality changes also in the blood but these are not indicated in the Fig. Note that the direction of blood flow may be reversed to that illustrated in the Fig. without any functional consequences for the proposed mechanisms.

pronounced *in vitro* and one may question the relevance of such *in vitro* studies for the situation *in vivo*.

The tissue hyperosmolality is probably not only of importance for transepithelial water transport but also for the movement of water *within* the villus. According to Curran's model, interstitial tissue pressure is increased in the hyperosmolar compartment. Hence, the osmolality gradient along the villous length is probably accompanied by a corresponding hydrostatic gradient which establishes a pressure head for the flow of lymph in the central lacteal. This explains why such a high percentage of the absorbed water (up to 50 %) seems to be absorbed *via* the lymphatics (Simmonds 1954, Barrowman and Roberts 1967, Jodal *et al.* 1975).

According to current concepts the absorption of glucose and amino acids across the luminal membrane of the enterocyte is in part driven by a sodium concentration gradient between lumen and cytoplasm (see e.g. Crane 1962). This gradient is maintained by the sodium pumps at the baso-lateral plasma membranes of the intestinal cell. The intracellular sodium concentration (Na_{ic}) is then assumed to be fairly low. Esposito *et al.* (1973), for example, proposed that Na_{ic} *in vivo* is about 20 mmol/l. The three models discussed above infer that Na_{ic} is at least 100 mmol/l. In fact, two of the models (A and C) implied that no sodium gradients at all exist between lumen and cell. It should be pointed out, however, that the calculated intracellular sodium concentrations may represent mean values. One may imagine an uneven sodium concentration within the cell, the concentration being higher at the basal than at the luminal membrane due to the large osmotic pressure present in

its full osmotic force 800 mOsm/kg H_2O would equal about 20 atm, or around 15 mm Hg. Since most of the hyperosmolality in the villi is probably made up by sod chloride, one has to know the reflection coefficient (σ) of this solute with regard to intestinal epithelium to be able to evaluate the magnitude of the osmotic forces invol Fordtran *et al* (1965) made an attempt to estimate σ for NaCl in the human small bo They arrived at values of 0.58 and 0.95 in the jejunum and ileum respectively There r however theoretical difficulties in determining σ for NaCl in the intestine since sodi actively transported by the enterocytes. Furthermore, ions are charged and the "wal of the intestinal pores are considered to be electrically negative Using a value of 0.6 a rough estimate of the intestinal σ for sodium chloride it can be calculated that an osm pressure head of about 9 000 mm Hg would be present across the epithelial lining at t villous tip In the upper third of a cat villus exposed to a Krebs-glucose solution it ca similarly calculated that an average osmotic pressure head of about 5 000 mm Hg (see Table I)

Such large pressure differences must be of great importance for water absorption. The fore in agreement with Curran s model for water absorption it is proposed that the vil hyperosmolar region, demonstrated in the present study represents the hyperosmolar co partment necessary for explaining intestinal water absorption *in vivo* This conclusi corroborated by the present observation that marked reductions of intestinal tis hyperosmolality *e g* by luminal perfusion with choline mannitol solutions (Fig. 5 and 6 turned the intestine into a secretory organ This observation may in turn, be of importa in explaining certain types of diarrhea. A more detailed experimental analyses of t relationship between water absorption and villous tissue osmolality will be presented i subsequent studies.

The magnitude of the osmotic forces involved *in vivo* during water absorption m imply that solvent drag plays a significant role in epithelial transport in the gut. This turn, has at least two important functional implications. — *First* according to Diam and Bossert (1967) the intercellular spaces constitute the site of "standing osmotic gradien proposed to be of great importance for water absorption. This hypothesis was origin proposed assuming that the luminal end of intercellular spaces was completely "tigh However recent experimental evidence strongly indicates that this is not the case in so epithelia including the gut (Frömter and Diamond 1972) Since the flow of fluid thro these paracellular spaces must be very high, indeed it is questionable if any gradients the type proposed by Diamond really exist *in vivo* in the intestinal epithelium. Furthermo a high concentration of extracellular sodium chloride is created at the "tissue end the intercellular space by the countercurrent multiplier which, in turn, tends to create intercellular concentration gradient of opposite direction to the one proposed by Diamo *Second* the presence of large unstirred layers in the intestinal lumen has been propo by *e g* Dietschy (1975) and these layers are believed to be of great functional significance *e.g* lipid absorption However such studies have been largely performed in *in vitro* org baths where increases in tissue osmolality created by "active" mechanisms are proba only a fraction of those present *in vivo* (*cf* ischemia, Fig. 7) Consequently the extent ... and convectional stirring in the lumen must quantitatively be ... ch

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the villous tissue, forcing water out of the cells at their basal end. This suggestion is [illegible]raborated by the uneven intracellular potassium concentration reported by Zeuthen [illegible] Monge (1975) Moreover the luminal concentration of sodium adjacent to the cell [illegible] higher than that of perfusate. This is suggested by the somewhat higher osmolality [illegible] in the lumen close to the cells (see Results) It is, however difficult to determine this [illegible]lality partly because of the different calibration curves for tissue and electrolyte [illegible] (see Fig. 2)

In textbooks of physiology it is usually proposed that villous structures, like those [illegible] in the gut are primarily designed to increase surface area in order to enhance the tran[illegible] capacity of various solutes (*cf* Wilson 1962) Most villous and papillary structures [illegible] however supplied with a vascular arrangement similar to that of the villi in the [illegible] intestine, implying the presence of a countercurrent exchanger It is therefore proposed [illegible] villous and papillary structures in many organs are designed mainly to act as coun[illegible] current multipliers, thereby primarily enhancing water transport. One may even [illegible] villi that "secrete" fluid due to a countercurrent multiplication of a *hypotonicity* (see Fig [illegible] when net solute flux is directed from plasma to lumen These proposals are substantiated [illegible] recent experimental findings that the filliform and fungiform papillae on the cat's [illegible] exhibit an osmolality gradient of the same type as in intestinal villi, tip osmolality [illegible] 800–950 mOsm/kg H_2O when exposed to a Krebs-glucose solution As in the small [illegible] this osmolality gradient is decreased by exposing the tongue to a choline-mannitol [illegible] (observations to be published) Admittedly the papillae of the tongue are not impor[illegible] for overall water absorption but the presence of a hyperosmolar tissue region [illegible] these villi like structures strongly suggests that the papillae of the tongue are supplied [illegible] the vascular arrangement and the "active" transport mechanisms necessary for an effec[illegible] countercurrent multiplication.

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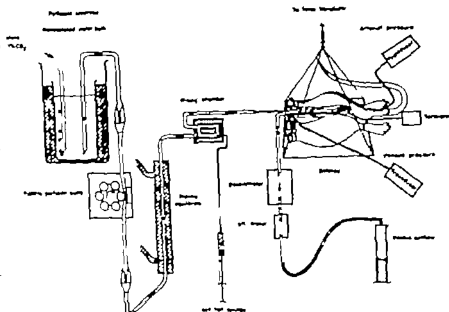
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Schematic dra ing illustrating the experimental arrangements.

e course of physiological and pathophysiological alterations in the perfused capillary ce area and/or in permeability. The mutual relationship between these two events, of importance for nutritional exchange and for the fluid equilibrium between blood and stitium, respectively, is incompletely understood and their simultaneous recording and parison is therefore of considerable interest for the full exploration of capillary function. rief description of the technique has earlier been presented (Rippe and Stage 1976).

Methodological procedures

General principle. Dye molecules were used as tracers. Cardio-Green (CG) (Hynson, stcott and Dunning Inc., USA), bound to albumin, was used as the non-permeant tracer d Cr-EDTA as the permeant one. The venous effluent was passed through densitometer tem, by means of which the concentrations of the two tracers could be separately and ultaneously recorded and hence directly visualized, allowing for rapid evaluation of the change events. The general experimental arrangements are shown in Fig. 1.

– *Preparation, perfusate.* Experiments were performed on the isolated hindquarter prepara on of the male Wistar albino rat, tail and paws excluded (*cf.* Folkow *et al.* 1974). It was rfused by means of a Harvard perfusion pump, using as perfusate gassed (97% O and 3% O_2) and warmed (38°C) Tyrode solution which contained 4% dextran (Macrodex 70, ndly supplied by Pharmacia, Sweden) and 0.5% horse serum (Normal serum, SBL, Swe en). The preparation, mainly consisting of skeletal muscle, was arranged for the "isogravi- metric technique" (Pappenheimer and Soto Rivera 1948) which allows frequent estimations f capillary filtration coefficient (CFC) and mean capillary pressure (Eliassen *et al.* 1974).

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An "on-line" colorimetric method for repeated, rapid determinations of capillary diffusion capacity

By

BENGT RIPPE and LARS STAGE

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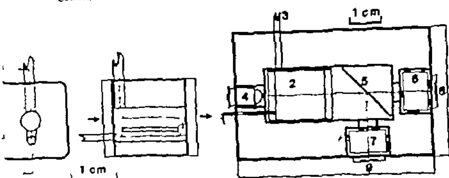
Abstract

RIPPE, B and L. STAGE. *An "on-line" colorimetric method for repeated, rapid determinations of capillary diffusion capacity* Acta physiol. scand 1978 *102* 108–115

The single injection indicator diffusion method for estimation of capillary diffusion capacity (Crone [...] has been developed to provide directly visualized, continuous colorimetric recordings of the venous [...] concentration curves by means of a two-colour densitometer system. Cardio-Green, bound to albumin [...] used as the non permeant ("reference") tracer and Cr EDTA as the permeant one. The artificially perfused [...] hindquarter muscle vascular bed of rats is used Highly reproducible curve recordings can be obtained [...] every fourth minute. Accidental disturbances of the recordings are readily detected. Thus, compared [...] fractional venous sampling technique, the present technique has the great advantage of allowing [...] controlled and frequently repeated determinations of capillary diffusion capacity It is therefore [...] together with measurements of filtration-absorption events, [...] for frequent quantitative comparisons [...] capillary diffusion and filtration capacities over a wide range of induced changes in perfused capillary [...] face area and/or capillary permeability The main limitation is that the employed permeant tracer [...] tates the use of erythrocyte-free perfusates.

The single injection indicator diffusion method (Chinard *et al* 1955 Crone 1963) [...] quently employed for investigations of the transcapillary diffusion exchange. The appli[...] bility and validity of this method has been discussed extensively earlier (*cf* Crone and L[...] 1970 Lassen and Trap-Jensen 1970 Bassingthwaighte 1974). Until now the venous [...] concentration curves have been obtained by fractional venous sampling, implying [...] numerous samples must be collected and subsequently determined with respect to tr[...] concentrations, to allow for a single analysis of capillary diffusion exchange. As this [...] fairly laborious procedure it is seldom possible to obtain more than a few such analyses [...] an individual expt. and for essentially the same reason there have been difficulties in co[...] bining the single injection technique with other techniques for functional capillary stu[...]

On this background the single injection technique was modified to make possible a d[...] visualization of the venous time-concentration curves, and thus allow for frequently [...] peated determinations that could be immediately checked. One of the main reasons was [...] [...] and quantitative comparisons of capillary diffusion and filtration ex[...]



Left part shows the cuvette part of the densitometer The broken line at right shows the flow path the large arrow the light path Right part shows the densitometer 1 inlet, 2, cuvette, 3 outlet, 4 ;, 5 double glass wall, 6 542 nm filter, 7 805 nm filter 8 and 9 photocells. The broken line shows the path

Sudden intraarterial tracer injections were performed by means of a precision step dis- ser delivering a constant amount of injectate in every bolus (40 $\mu l \pm 0.4\ \mu l$ mean $\pm$ S.D). is means that with every injection 6–8 moles of CG-albumin and 3–4 micro moles of -EDTA entered the vascular bed. The injectate was delivered to the perfusate inflow minute mixing chamber that efficiently mixed the injectate with the perfusate.

3 *pH measurements.* pH of the venous effluent was measured continuously to correct for pH dependence of the permeant tracer curve. A pair of ordinary pH electrodes placed the effluent from the densitometer cuvette were used. pH was recorded on one channel of a entiometer writer via an amplifier unit.

The pH recording channel was also used to mark the start and end of each tracer injection means of an automatic switch in the step dispenser coupled to the pH amplifier unit. us was performed to allow measurements of mean transit times.

6 *Recording devices and curve recordings.* The small recording cuvette (volume about 3 ml) is shown in Fig. 2 left part, and the cuvette-densitometer unit in the right part of g 2 In the cuvette light passed through 16 mm of the perfusate. It is of great importance at the contents is well stirred. This was achieved by an arrangement inside the cuvette that lowed minor recirculation and hence a stirring effect, where the inlet provides the driving rce The outflow resistance was moderately increased by the stirring device which, how- er in the experimental situation was compensated for by keeping the venous outflow funnels up lowered to avoid undue increases in capillary pressure.

In the right part of Fig. 2 the mirror 5 is a double glass wall which splits the light beam the two filters (6 for Cr-EDTA and 7 for CG), which are of the interference type with peak transmissions at 542 and 805 nm, respectively (DAL, ZEISS West Germany). Non- nearity in the CG recordings may occur with some CG light filters. The reason for this is a ight difference between the CG absorption peak and the peak of transmission of the light lter

Photocells 8 and 9 are of type BPW33 (Siemens) and their outputs are connected to two mplifiers as shown in Fig. 3 The input part to the left acts as a current-to voltage amplifier The subsequent part is balanced logarithmic amplifier with two BC239 acting as log-

For such purposes the preparation was continuously weighed, and arterial pressure (in a artery) and venous pressure (in sidebranch of caval vein) could be set to any desired level In addition flow could be varied by adjusting the setting of the perfusion pump. Venous outflow was repeatedly collected directly in a graduate cylinder tube to check the setting of the pump Flow was always kept above 11 ml/min 100 g muscle tissue to eliminate the risk for significant flow limitation of transport This was checked by repeated determinations of diffusion capacity over a wide range of flows. Flow dependence of diffusion capacity was only evident at flows below 10 ml/min 100 g of tissue.

This *in vitro* preparation has the great advantage that it can be utilized both for studies during maximal vasodilatation, allowing analyses of capillary events during perfusion of the entire capillary surface at normal or altered permeability and during exactly graded vasoconstrictions, or vascular microplugging, implying alterations also of the perfused capillary surface area. For direct quantitative comparisons of diffusion and filtration events it is obviously of great importance that essentially all other relevant hemodynamic parameters can be closely controlled

3 *Properties of the tracer molecules* When bound to albumin CG (m w 775) has a strong and narrow absorption peak at 805 nm and practically none at 542 nm, where Cr EDTA has its strongest absorption One disadvantage is that diluted CG is relatively unstable, but its absorption decreases only 2–3 per cent during the few hours needed for the experiment, which can easily be corrected for

Cr EDTA (m w 341) has its strongest light absorption at 542 nm and almost no absorption at 805 nm Its light absorption at 542 nm is somewhat dependent of pH However in the pH-range of use (6 6–7 4) correction can easily be made since the absorption here decreases by about 2% per 0 1 unit of pH increase.

In this type of colorimetric recording, Cr EDTA is given in considerably higher concentrations than when radioactive Cr EDTA is used To exclude significant admixture of free cromium ions or free EDTA in the injectate, which might have toxic effects respectively bind calcium ions, the following procedures were used 0.2 molar solutions of CrCl and Na-H EDTA were mixed with CrCl in slight excess. The mixture was boiled, allowed to cool and pH was adjusted to 7 3 with NaOH The solution was left for 24 h to allow excess cromium to precipitate as cromium hydroxide and was then filtered. It could be calculated that a 0 1 mol/l Cr EDTA solution, produced according to these principles, has a Cr concentration as low as about 10⁻ mol/l and free" EDTA about 10 mol/l Since each tracer injection contained only 3–4 micro mol Cr EDTA the amounts of Cr and free EDTA that reached the tissues were negligible.

4 *Tracer injections.* The injectate contained 70–100 mmol/l Cr EDTA 0 –0.3 mmol/l CG 6–10 mmol/l albumin (Human Serum Albumin Kabi Sweden) and about 250 mmol/l NaCl The albumin content eliminated the possibility that free CG could reach the preparation.

The injectate was hyperosmolar (6–800 mosmoles/l) mainly as a result of the way Cr EDTA was prepared (see above). However because of the minute amounts injected (40 µl) in relation to flow (> 10 ml/min) no detectable osmotic transient was created, as judged from the continuous weight recordings.

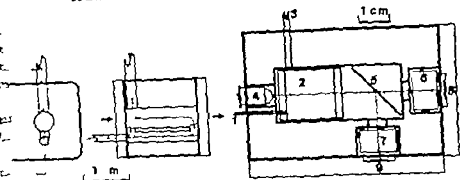


Fig. 2. Left part shows the cuvette part of the densitometer. The broken line at right show the flow path and the large arrows the light path. Right part shows the densitometer: 1 inlet, 2 cuvette, 3 outlet, 4 lamp, 5 double glass wall, 6 542 nm filter, 7 805 nm filter, 8 and 9 photocells. The broken line shows the light path.

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6. Recording devices and curve recordings. The small recording cuvette (volume about 3 ml) is shown in Fig. 2 left part, and the cuvette-densitometer unit in the right part of Fig. 2. In the cuvette light passed through 16 mm of the perfusate. It is of great importance that the content is well stirred. This was achieved by an arrangement inside the cuvette that allowed a minor recirculation and hence a stirring effect, where the inlet provides the driving force. The outflow resistance was moderately increased by the stirring device which, however, in the experimental situation was compensated for by keeping the venous outflow cannula tip lowered to avoid undue increases in capillary pressure.

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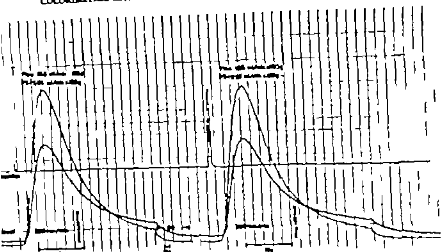
Fig. 3 Schematic diagram of the photocell amplifier The amplifier CA3130 is used as current to vol amplifier and the two 741 form a logarithmic amplifier F7805 is oltage regulator

arithmic elements. The transistors have to be thermally tied together The outputs ar recorded on two channels in a three-channel potentiometer recorder (Rika Denki, mod B34 Full scale deflection time <1 s) Actual recordings of venous time-concentration cur are shown in Fig. 4

Calibration of the densitometer measuring system was performed by aspirations of pe fusate samples, containing known concentrations of the injectate, through the cuv system, where the pH measuring device automatically gives the pH of the solutions

7 *Analysis by calculator* The analysis of the tracer curves was made in a calculator sys (Hewlett Packard 9820A). The curves were sampled and entered into the calculator va digitizer unit (HP 9864A) In the first computation step the possible nonlinearity of the C recording, as well as the pH dependence of the Cr EDTA recording, was corrected for an the curves were standardized in relation to the calibration recordings, mentioned abov The traced curves were then plotted by a calculator plotter in a normalized form with resp to peak CG concentration (Fig. 5)

In the subsequent computation step areas under the tracer curves were estimated in ord to obtain approximate values for mean transit time and flow Since the single injection cur for diffusion estimations were usually registered for only the first minute, estimations mean transit time and flow necessitated that the curve tails were either extrapolated, or th entire curve recordings were performed The entire curve recordings showed that the trac concentrations did generally not reach zero levels until after 3–4 min, and further a lo arithmic plotting revealed that both the permeant and the non-permeant indicator cur tails were multiexponential (Fig. 5).



4. Original recording of tw consecutive venous time—concentration curves during constant hemo-mic conditions. I this case the injectate calibration value for CG-albumin equalled that for Cr-EDTA. pH recording for the venous effluent is also shown, with the superimposed injection markings.

he apparent fractional extraction function, E(t), is defined as:

$$E(t) = \frac{Cn(t) - Cp(t)}{Cn(t)}$$

re Cn is the standardized concentration of the non-permeant tracer and Cp that of the rmeant one at time t, and E(t) was computed and plotted (Fig. 5). E varies with time, anly because of nonuniformity in design of capillary network and in flow interlaminar d longitudinal diffusion between the permeant and non-permeant tracer and back fusion (*cf* Crone and Lassen 1970). To get a weighted mean of the extraction occurring *all exchange* vessels, *i.e.* the "short" ones with low extraction as well as the "long" ones th high extraction, the overall extraction was calculated according to the formula

$$E(T) = \frac{\int_{-0} Cn(t) - \int_{-0} Cp(t)}{\int_{t=0}^{T} Cn(t)}$$

E calculated in this way *i.e.* by means of the "area method" was plotted as a function of different integration end points (T-values) and formed a plateau at T-values between peak concentration time of the tracer curves and the time where they had declined to about 50% of peak value (Fig. 5).

This plateau value for E was used in the calculations of the capillary diffusion capacity or PS-product (Renkin 1959) according to the expression PS = -Qp ln (1-E) where Qp is the directly measured perfusate flow assuming monoexponential concentration profile in the exchange vessels.

8 Experimental errors. When all hemodynamic parameters were kept constant the random

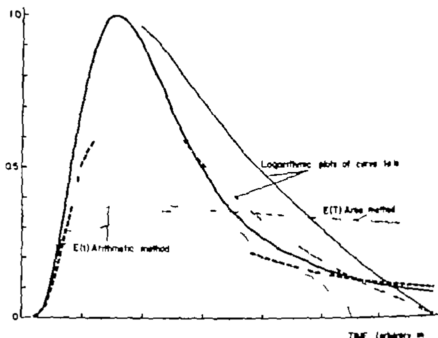


Fig. 5 Computer diagram fo the tracer curves shown to the right in Fig. 4. The curves are here standard to the injectate calibration and plotted in a *normalized* form with respect to peak CG concentration. The curve *tails* are also plotted logarithmically. Note their multiexponential decay. In addition, the "appar fractional extraction E (t), as well as the "integrated (area) extraction E(T) are shown. The follow computed data were obtained from the original recordings. E(T) (area method) t plateau-time PS = 5.92 ml/min 100 g flow computed from the CG curve = 14.58 ml/min 100 g (directly measur flow = 13.50 ml/min 100 g) mean transit time for CG = 15 6 s and for Cr EDTA 2…8 s. Flow and me transit times were here estimated after monoexponential extrapolation from the portion here the C curve had decreased to 10 per cent f it peak concentration.

experimental error determined as the *coefficient* of variation between consecutive PS deter minations in 25 expts was less than 3 per cent in a given expt (*cf* Fig. 4). Variations betwe individual preparations, was of the order of 10 per cent, which includes experimental err and biological variation

As regards estimation of flow and mean *transit times* from the curves, systemic err were induced by using monoexponential extrapolation of the multiexponential curve ta (*cf* Fig. 5). If however the extrapolation was performed after the curves had declined t 10% of peak value, computation of *flow from* the CG curve gave a systemic overestimat of this parameter of only 5–10%. The error becomes *much* greater if mean transit time calculated from the extrapolated curve instead of *from* the total curve, registered for 3–4 m simply because the end of the tails can be *quite* long as a result of the multiexponential dec Therefore, in circumstances where *mean transit* times are to be used for experimental pu poses, it is advisable to record the total curve, *i.e.* for 3–4 min.

The presence of a multiexponential decay is not *surprising* since even a *fairly* "uniform skeletal muscle contains portions (*e.g.* "tonic" and *phasic*" muscles) that differ somewh in their vascular arrangements both in terms *of flow* conductance and capillary exchan area. Further in some parts of the preparation, *circulation* may have been somewhat di turbed due to tissue ligatures, *etc*

Comments

[Th]e main advantage of this modification of the indicator diffusion method is that a great [n]umber of recordings can be frequently performed with high precision and reproducibility [an]d under direct visual control. Consequently accidental interferences can in most cases be [im]mediately detected from the curve characteristics. It further allows repeated recordings [be]fore, during and after induced, changes of the hemodynamic situation, whether brought [ab]out "passively" by changes in pressures and flows or by "active" changes in vascular [s]mooth muscle activity and/or in endothelial permeability. Furthermore, the recordings of [d]iffusional transport can readily be associated with concomitant determinations of the [f]iltration-absorption and of the hydraulic conductivity in the capillary section (CFC mea[su]rements). Series of such experimental studies have been performed and will be presented [in] subsequent publications. Lastly it might also be possible to use diffusible tracers of dif[fe]rent molecular weights, for repeated estimations of restricted diffusion effects.

The method has one obvious drawback in the use of Cr-EDTA as the diffusible tracer [fo]r colorimetric recordings makes it necessary to use cell-free perfusates. It might, however [n]ot be impossible to find a suitable diffusible indicator which, like the non-permeant CG [tr]acer can be used also during ordinary blood perfusion. This problem has not been ap[p]roached so far since the method was developed primarily to be used during some basic [e]xperimental situations, where parameters like pre and postcapillary resistances, vascular [t]one, pressures, capillary surface area etc. could be quantitatively controlled, and then an [in vi]tro system is preferable.

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AB Hässle generously covered part of the expenses for technicians.

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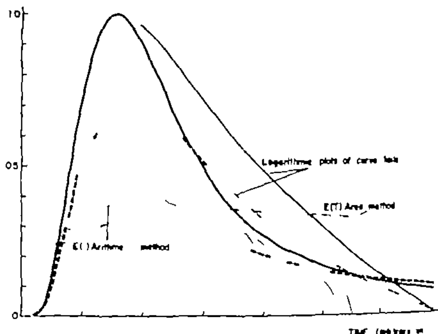


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ABLE I Dry weight (acetone dried powder) and choline acetyltransferase activity of ventricles in untreated rats and in rats treated ith chlorisondamine. Values are mean ± S.E. Number of observations indicated in brackets.

	Dry weight mg	Enzyme activity	
		In μg ACh/h/ventricles (total activity)	I μg ACh/h/g acetone powder (concentration)
lorisondamine	89.7 ± 2.2 (9)[a]	16.3 ± 0.9 (9)[a]	180.6 ± 7.4 (9)[b]
ontrol	113.2 ± 2.2 (9)	22.1 ± 0.7 (9)	195.7 ± 5.8 (9)

0.05   0.001 When the ventricles of the treated rat are compared with those of the control litter mate

f choline acetyltransferase was determined according to the method of Catherine Hebb (see Nordenfelt 963). Of the tissue extract 0.4 ml was incubated for 1 h at 38°C. The acetylcholine formed was estimated on e frog rectus muscle and expressed in μg acetylcholine chloride formed per h per ventricles of each heart otal activity) and in μg acetylcholine chloride formed per h per g acetone powder (concentration); the tissue xtracts from the drug treated rat and its untreated litter mate were incubated simultaneously and the incubates were assayed on the same rectus muscle.

For comparison report on the activity of choline acetyltransferase in ventricles of rats given the β-drenoceptor stimulating drug isoprenaline sulphate has been included. The ventricles ere analysed in onnection with study on salivary glands (Ekström 1974). Of the drug 3 mg in 0.2 ml saline was given c. very 8 h for 21 days. The rats were of the same sex, age, weight and strain as those given the ganglion locking drug and further, the ventricles were treated as outlined above.

For statistical analyses Student' t-test was used, paired comparisons were made between the treated rat nd its untreated litter mate. P-values less than 0.05 were considered significant.

Results

Treatment with chlorisondamine. At the start of the experiment no difference existed in body-weights between the rats to be given the drug and those to be used as controls. At the end of he experimental period the drug treated rats were found to have lost weight, while the weight of the control rats had remained unchanged, expressed as a percentage the body eights of the drug treated rats were (mean ± S.E.) 82.3 ± 1.0% (n = 9) of those of the untreated rats (p 0.001). The dry weights (in acetone dried powder) of the ventricles in the drug treated rats were lower (p 0.001) than in the untreated ones (Table I)· the weight of the muscle in the treated rats being 79.1 ± 0.8% (9) of that in the untreated rats (Fig. 1). The total activity of choline acetyltransferase in the ventricles of the treated rats was 73.4 ± 3.0% (9) of that in the controls the difference in total activity between the two groups of animals as significant at the p 0.001 level. When the activity of the choline acetyltransferase as expressed in terms of concentration no significant (0.05 p < 0.1) difference was found between treated and untreated animals.

Treatment ith isoprenaline. Since both the dry weight and the total activity of choline acetyltransferase of the ventricles were reduced after the treatment with chlorisondamine (resulting in the unchanged enzyme concentration), the question arose whether a change in

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Fall in choline acetyltransferase activity in the ventricles of the rat heart after treatment with a ganglion blocking drug

By

JÖRGEN EKSTRÖM

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Abstract

EKSTRÖM, J *Fall in choline acetyltransferase activity in the ventricles of the rat heart after treatment with a ganglion blocking drug* Acta physiol scand. 1978 *102* 116–119

The choline acetyltransferase activity was found to be 27% lower in the ventricles of rats which had been given the ganglion blocking drug chlorisondamine than in those of untreated rats. Since the weights of ventricles were also lower in the treated rats than in the untreated ones, it was wondered whether a change in the muscle mass as such affected the activity of the enzyme: isoprenaline treatment was found to cause the weight to increase markedly while it left the enzyme activity unchanged. The decrease in enzyme activity observed after the prolonged ganglion blockade is interpreted as being the consequence of a reduced traffic of impulses along the postganglionic parasympathetic nerves of the ventricles. Thus, the results of the present investigation give further support for the idea of a vagal innervation of this part of the heart.

Activity of the acetylcholine forming enzyme, choline acetyltransferase, has been demonstrated in the ventricles of the rat heart (Ekström 1970). This finding seems to support the idea of a parasympathetic innervation of the mammalian ventricles (see DeGeest et al. 19.. and Higgins, Vatner and Braunwald 1973). In a recent investigation (Ekström and Lindmark 1977) the choline acetyltransferase activity was found to be reduced in the postganglionic parasympathetic nerves in the parotid glands of rats after prolonged treatment with a ganglion blocking drug. The decrease in enzyme activity is attributed to a reduced traffic of impulses along these nerves (see Ekström 1975). In the present study the effect of ganglion blockade on the choline acetyltransferase activity of the ventricles is described.

Methods

Female rats, 3–5 months old and weighing about 200 g, of a Sprague-Dawley strain bred at this Institute were used. The ganglion blocking drug chlorisondamine chloride was given in 0.2 ml saline s.c. every ... over a period of 13 days. The dose was gradually increased, starting with 1.5 mg per injection and ending with 3 mg; the total amount given of the drug being 81.5 mg. Untreated litter mates served as controls to the drug treated rats. The rats were killed with ether the drug treated animals 9–20 h after the last injection ... hearts were removed and the atria separated from the ventricles. Acetone dried powder was then ... from the ventricles. The powder was made up in cysteine saline in a concentration of 50 mg/...

Discussion

e present study shows that prolonged treatment with a ganglion blocking drug lowers the al activity of choline acetyltransferase in the ventricles of the rat heart. It is of interest to te that the reduction in enzyme activity observed was of the same magnitude, expressed as percentage, as that found in the parotid glands of these animals (Ekström and Lindmark 77). As pointed out by these authors the fall in the choline acetyltransferase activity of the stganglionic nerves of the parotids caused by the drug was probably a specific effect and t due to a general toxic action on cholinergic nerves, since the activity of this enzyme was t reduced in the preganglionic sympathetic nerves of the adrenals (Ekstrom and Lindmark 77). After the treatment with the ganglion blocking drug the weight of the ventricles was und to be lower than in untreated animals. The percentage fall in ventricular muscle weight as of about the same magnitude as that in body-weights. The outcome of the expt. using oprenaline indicates that a change in the muscle mass as such is not accompanied by a milar change in choline acetyltransferase activity- this is an observation in agreement with udies on skeletal muscles (Diamond, Franklin and Milfay 1974) and salivary glands kstrom 1974).

It seems reasonable to interprete the decrease in choline acetyltransferase activity in the entricles which was found after the treatment with the ganglion blocking drug as being a onsequence of a reduced flow of impulses in the postganglionic parasympathetic nerves. his was also the suggested explanation of the fall in the enzyme activity in the parotid lands (Ekstrom and Lindmark 1977). Thus, the results of the present investigation seem to ve further support for the concept of a vagal innervation of the ventricles of the heart.

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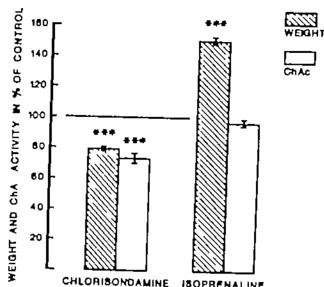


Fig. 1 Dry weight and choline acetyltransferase activity per ventricles of treated rats either with chlorisondamine or isoprenaline and of their corresponding litter mates. The weight and the enzyme activity are expressed as percentage of those of the ventricles of the control litter mate (mean ± S.E.). Number of comparisons in the experiment with chlorisondamine is 9 and in the one with isoprenaline 6. *** indicates a P value less than 0.001.

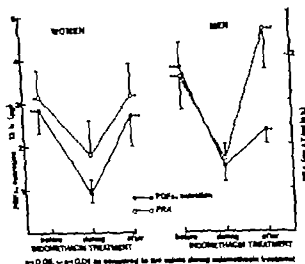
the ventricular mass in itself would affect the total choline acetyltransferase activity. Prolonged treatment with isoprenaline has been found to cause the ventricular weight of rats to increase (Schneyer 1962), a finding which was confirmed (unpublished) in connection with a study on salivary glands (Ekström 1974). The effect of this drug on the weight and choline acetyltransferase activity of the ventricles is now reported.

Neither at the start nor at the end of the experimental period was there any significant difference in body weights between the animals given the drug and their untreated litter mates. The dry weights of the ventricles were markedly increased ($p < 0.001$) after the treatment; as a percentage the ventricles of the isoprenaline treated rats weighed 149.6±2.4 ($n = 6$) of those of the controls (Table II, Fig. 1). The total activity of choline acetyltransferase in the ventricles of the treated rats was about the same as that in the ventricles of the animals serving as controls. In percent it was 96.7 ± 1.6 ($n = 6$) when ventricles of the treated rats were compared with those of the untreated ones. Consequently, the concentration of choline acetyltransferase was lower ($p < 0.001$) in the ventricles of the treated rats than in those of the untreated rats.

TABLE II. Dry weight (acetone dried powder) and choline acetyltransferase activity of ventricles of untreated rats and in rats treated with isoprenaline. Values are mean ± S.E. Number of observations is indicated in brackets.

	Dry weight mg	Enzyme activity	
		In µg ACh/h/ventricles (total activity)	In µg ACh/h/g acetone powder (concentration)
Isoprenaline	191.9 ± 6.3 (6)[a]	23.4 ± 1.9 (6)[b]	121.7 ± 7.0 (6)[a]
Control	128.3 ± 3.8 (6)	4.. ± 1.6 (6)	188.3 ± 10.8 (6)

[a] P < 0.001 When the ventricles of the treated rats were compared with those of the control litter mate.



g 1 The effect of indomethacin treatment on the prostaglandin $F_{2\alpha}$ and plasma renin activity in 9 women and 12 men with essential hypertension The values are mean ± S.E.

women (r 0.532, p 0.05) and men (r=0.613 p<0.01) The inhibition of PG synthesis has been documented to reduce glomerular filtration rate and Na excretion in some studies (Kampl *et al* 1975 Donker *et al.* 1976), whereas this has not been verified in all papers (...ree *et al* 1976). In the present study treatment of 6–7 days with the conventional indomethacin dosage did not suppress the urine volume and electrolyte excretion (Na and K).

Our results indicate that there is a close correlation between the decreases of PG excretion and PRA in patients with essential hypertension during the indomethacin treatment. In contrast to hypertensive animals (Schölkens and Steinbach 1975 Levy 1977), the rise of BP in man was relatively slight. This may be explained by the fact that the decrease in the production of PGs, many of which have vasodilatatory effects, is compensated by a concomitant decrease in PRA. An increase of this magnitude in BP is not of marked importance in slightly hypertensive patients. On the other hand, in patients with severe hypertension the compensatory mechanisms are further impaired, and the pressor action of prolonged treatment with anti-inflammatory analgesics might be of practical significance.

Table I Blood pressure before, during and after the treatment with indomethacin (75 mg daily for 6–7 days) in 9 women and 12 men with essential hypertension. The values are mean ± S.E.

	BP	1 2 weeks[a] before	Indomethacin treatment		
			1 day before	6–7th day during	1 week after
Women	syst.	176 _ 9	177 ± 9	183 ± 9	168 ± 9**
	diast.	113 ± 3	114 ± 3	113 ± 3	109 ± 2*
Men	syst.	163 ± 4	158 ± 6	156 ± 5	154 ± 5
	diast.	113 2	111 ± 2	112 ± 2	107 ± 2*

[a] The lowest values during the "run-in" period
* p 0.05 and ** p 0.01 ...

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Dependence of plasma renin activity on prostaglandin excretion in essential hypertension

By

PAULI YLITALO HEIKKI VAPAATALO, TIMO METSÄ KETELÄ and TIMO PITKÄJÄRVI

Indomethacin decreases prostaglandin (PG) synthesis and plasma renin activity (PRA) experimental animals (Schölkens and Steinbach 1975 Romero *et al* 1976) and also in ma (Patak *et al* 1975 Rumpf *et al* 1975) A slight simultaneous increase in blood pressure (BP has been found Thus, inhibitors of PG synthetase cause a sensitization to some factors t elevate BP (Lee *et al* 1976 Negus *et al* 1976) In man, this elevation of BP has been co nected to the decrease in group A of circulating PGs. However no clear marked corre tion between plasma prostaglandin A and any changes in renal function or BP could found (Patak *et al* 1975 Lee *et al* 1976) We can now report a close correlation betw the decreases in PRA and in PG urinary excretion caused by PG syntethase inhibitor both female and male patients with essential hypertension Special attention was direc to the alteration in BP and the urinary excretion of electrolytes was also measured

Twenty-one untreated, otherwise healthy patients (9 women and 12 men, aged from 40 to years) with recently diagnosed essential hypertension were treated daily with 75 mg (25 a.m and 50 mg p.m.) of indomethacin (Indetrit® Medica Ltd. Helsinki) for 6–7 days I was measured in supine position by a brachial cuff and a mercury manometer The diastol pressure was recorded at the fourth Korotkoff's sound. Before the measuring of BP t subjects rested in bed for 10 min The mean of 3 consecutive values was taken into acco $PGF_{2\alpha}$ and PGE were measured by radioimmunoassay according to the method of Ger man *et al* (1972) in 12 h urine collected overnight. For the radioimmunoassay of a metabol of $PGF_{2\alpha}$ (13 14-dihydro-15-keto-PGF) commercial kits (Clinical Assays Inc., Cambri Mass.) were used Urinary Na and K were determined by a flame photometer and PR by the radioimmunoassay of A I released in the incubation as described by Fyhrquist *et al* (1976)

BP tended to increase during the indomethacin treatment The difference was signific when BP after drug withdrawal was compared to that during the indomethacin treatme with the exception of systolic BP in males (Table I) $PGF_{2\alpha}$ excretion was suppressed 1/2–1/4 from the initial levels by indomethacin (Fig. 1) There was a similar decreas tendency in $PGF_{2\alpha}$ metabolite and PGE excretions during indomethacin treatment. The tim concentration curve of PRA followed closely the $PGF_{2\alpha}$ excretion. Significant correlati between the decreases caused by indomethacin in these parameters were calc ted in bo

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Binding of ^{3}H 5-hydroxytryptamine to synaptic plasma membranes of rat brain

By

J. GRIPENBERG and S.-E. JANSSON

…dying the binding of neurotransmitter substances and drugs to membrane preparations … the central nervous system is a new approach to examining receptor functions. However …st such studies have been performed using long incubation times, solutions lacking …ential cations and with crude membrane preparations which have been stored frozen for …ng periods or at 0°C (Seeman *et al.* 1975, Bennet and Snyder 1976, Fillion *et al.* 1976). …e use of less physiological conditions may distort agonist-receptor interactions and lead … conclusions not necessarily valid *in vivo*. Using a rapid filtration technique and short …ubation times we have studied the binding of ^{3}H-5-hydroxytryptamine (^{3}H-5-HT) to …aptic plasma membranes of rat brain and compared the time course of the binding in an …tonic electrolyte solution with that in a buffer solution devoid of essential cations. We … report on the effect of storage of the membrane preparation and preliminary results … the saturation characteristics of the binding reaction.

…ague-Dawley rats (200 g) were decapitated, the brains immediately removed and the cerebelli discarded. …er mincing in ice-cold 0.3… mol l^{-1} sucrose supplemented with 0.5 mmol l^{-1} EGTA, the material was …ogenized according to Gray and Whittaker (1962). After centrifugation (1 000 g, 5 min) and one wash …he sediment, the pooled supernatants were spun down at 15 000 g for 15 min, the pellets resuspended … 0.32 mol l^{-1} sucrose (3.0 ml g^{-1} original tissue) and layered on top of … gradient made up of 12 ml 5 % … 1.8 ml 11 … Ficoll (Pharmacia, Sweden) in 0.32 mol l^{-1} sucrose. After centrifugation at 21 000 g for 40 … he synaptosomes at the 5–11 … interface were collected, washed with sucrose and resuspended for … 5 mmol l^{-1} Tris-HCl buffer pH 8.1 for 60 min at 0°C with stirring. Synaptic plasma membranes … then isolated from the lysate by sedimentation-flotation centrifugation according t… Jones and Matus … at 60 000 g for 100 min. The membrane fraction was washed … with 20 mmol l^{-1} Tris-HCl, pH 7.40 … after sedimentation at 21 000 g for 30 min resuspended in … small volume of Tris-HCl and used …mediately or stored for 4 h at 0°C under nitrogen. The yield averaged 0.5 mg protein g^{-1} original tissue. …inding assays aliquots of the membrane suspension …ere added to 20 or 30 mmol l^{-1} Tris-HCl buffer … 40 or to … medium of the following millimolar composition: NaCl, 134, KCl, 5.6, CaCl$_2$, 2.2, MgCl$_2$, … NaH$_2$PO$_4$/Na$_2$HPO$_4$, 1.3, Tris-HCl, 20.0 pH 7.40 at 37°C. The protein concentration was 0.1–0.2 mg … After preincubation at 37°C for 10 min …th or … hout 5-hydroxytryptamine (5-HT) at 10^{-4} mol l^{-1} … 5-HT (sp. … 27.6 Ci mmol^{-1}, New England Nuclear) …as added to 1.0–3.5 ml of the membrane suspen- … Samples were taken at various time intervals, vacuum filtered through Whatman GF/A filters (⌀ 2.5 … and rinsed …th 20 ml of the respective medium. The …ashing … as completed in 10 … and the medium … kept cold in order to minimize dissociation of bound ^{3}H 5-HT. Specific binding was defined as the …al binding minus the binding occurring in the presence of 5-HT at 10^{-4} mol l^{-1}. Radioactivity trapped on … filters …as measured in … Wallac Decem NTL 314 liquid scintillation counter in … ylene based solution …derson and McClure 1973) at 34 % … efficiency. Proteins were measured by the method of Lowry *et al.*

This study was supported by a grant from Orion and Medica Scientific Foundation, Helsinki, Fel. We are grateful to the Minerva Institute for Medical Research, Helsinki, Finland, for the measurements of plasma renin activities.

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...sed logarithmically as the abscissa, the linearly presented specific binding fitted a sig-...oidal curve (Fig. 1 b). The Scatchard plot (Fig. 1 b, inset) of the 10 s binding values showed ...ature and assuming that only one population of binding sites is involved, this is sug-...tive of positive cooperativity. The corresponding plot of the 60 s binding values, on the ...er hand, fell on a straight line without any evidence of cooperativity. At 5 10^{-10} mol l^{-1} ... amount bound in 10 s was 38 % of that in 60 s, while at 3 10^{-9} mol l^{-1} the corresponding ...centage was 71. The specific binding taking place in 10 or 60 s both show saturability ...d both give a maximum of binding sites of about 250–300 fmol mg^{-1} protein but differ ...respect of the shapes of the saturation curves and Scatchard plots. These preliminary ...ults show that the electrolyte composition of the incubation medium, the incubation time ...d the condition of the membrane preparation markedly affect the binding between 5-HT ...d its proposed receptor. These factors should be taken into consideration when studying ...pharmacology and binding characteristics of the receptor.

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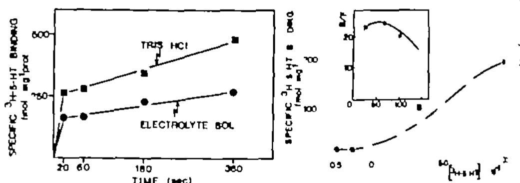


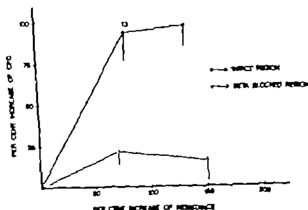
Fig. 1 a. Time course of specific binding of H 5-HT to rat brain synaptic plasma membranes. The centration of H 5-HT was 10^{-8} mol l⁻ ■ 20 mmol l⁻ Tris-HCl buffer ● electrolyte solution (se Each point is the mean of 5 expts.

Fig. 1 b Specific binding of H 5-HT to rat brain synaptic plasma membranes. The binding occurr 10 s was assayed as described in text. The amount of H 5-HT expressed in fmoles plotted against the centration of the amine in the medium as the logarithmic abcissa. The inset presents the data in a Sca plot, where B denotes the specific binding in fmol mg⁻ protein and F is the concentration of ³H 5-H fmol l⁻ Each point is the mean of 4–6 expts.

(1951). The morphology of the synaptosomal and synaptic plasma membrane fractions as checked Philips 300 EM electron microscope after glutaraldehyde fixation and using conventional techniqu specimen preparation. All chemicals used were analytical grade products available commercially

Imipramine at 3 10^{-6} mol l had no effect on the binding of H 5-HT to the syn plasma membranes. In 3 expts. with imipramine the binding recorded in samples taken tween 0 and 10 min of incubation in the electrolyte medium differed less than 5 fr controls. Thus, an operative presynaptic uptake mechanism for 5-HT in the prepar used seems unlikely even in the medium containing sodium. The time course of the spe binding of H 5-HT at 10^{-8} mol l and 37°C was different in Tris-HCl buffer 20 or 50 m l from that in the electrolyte medium (Fig. 1 a) In both mediums, however the bin consisted of a fast initial phase completed within 20 s and a slow late phase. The spe binding of H 5-HT in the electrolyte medium was lower and amounted to 59 1 52.1 5 and 60 4% of the binding in 20 mmol l Tris-HCl buffer after 20 60 180 and 360 s spectively (mean values, n = 5) This difference was evident only in fresh preparation storage for 24 h at 0°C under nitrogen did not affect the specific binding in Tris-HCl bu but in the electrolyte medium the specific binding was 47% above that observed in a fr preparation and only 14 % below that in Tris-HCl buffer (mean values, n = 3). Thus, h the ionic composition of the medium and the storage of the membranes affected the spe binding of H 5-HT Sodium ions might decrease agonist (5-HT) binding, i.e. 5-HT r have a higher affinity for a nonsodium conformation of the receptor as has been repor for the opiate receptor (Pert and Snyder 1974) Alternatively Tris-HCl might non-specific alter the conformational structure of the membranes unveiling potential 5-HT binding s which remain hidden in a physiological electrolyte environment

The fast initial specific binding of H 5-HT occurring in 10 s did not increase linearly a function of amine concentration and half maximal binding amounting to some 100 f fmol mg⁻ protein was achieved at 5 10 mol l When the amine concentration was c



1 Diagram showing the effect of noradrenaline infusion on CFC (...apillary sphincter tone) in ...tal muscle before and after ...ckade. The data are classed ... regard to the concomitant ...ase of vascular resistance and ... as mean value ± S.E. ... de... the number of observations

...scle upon the pattern of resistance response to noradrenaline was similar but the ...itude of the resistance increase was somewhat more pronounced for a given dose ... 25).

...he capillary filtration coefficient (CFC), reflecting the number of patent capillaries and, ...ce, precapillary sphincter tone, was determined in the control state before each period ... noradrenaline administration and after 2 3 min of infusion when the evoked resistance ...ponse had reached a steady state. Control CFC was quite stable in the individual ...mal and was unaltered by β-blockade: in the whole material control CFC varied ...ween 0.011 and 0.017 ml/min 100 g mmHg, the mean value being 0.014 ± 0.001 (S.E.) ...min 100 g mmHg (n 47). Noradrenaline administration to the intact muscle region ...ays caused a dilatation of the precapillary sphincters as evidenced by a clearcut increase ... the CFC. The CFC increment above control level ranged between 45 and 160 per cent ...l showed no consistent relation to the amount of infused noradrenaline. After β-blockade, ...ncrease of CFC was much reduced or abolished and in several of the infusion experi...nts a decrease of the CFC was instead observed. Fig. 1 summarizes the CFC alterations ...ring noradrenaline infusion (classed mean values ± S.E.), expressed in per cent of the ...trol values prior to infusion and plotted against the concomitant per cent increase of ...ional vascular resistance (increase of resistance by 40–100 and 100–200 per cent, ...pectively). It can be seen that the mean increase of CFC in the intact muscle region was ...out 100 per cent at the moderate as well as at the more intense constriction of the ...istance vessels. In the β-blocked muscle region there was also an increase of the CFC, ... the average, but the increment was much smaller (p 0.001) and amounted only to 19 ...d 16 per cent, respectively.

Comments

...e significant effect of blood-borne noradrenaline on peripheral vascular functions is ...nsidered to be vasoconstriction caused by α-adrenoceptor activation. Vasodilatation due ... activation of β-adrenoceptors has thus been regarded of little importance since, in the ...sistance vessels, the β-adrenergic inhibition of vascular tone is greatly overwhelmed by

Acta physiol scand. 1978, 102, 126–128

From the Department of Physiology and Biophysics, University of Lund, Sweden

Noradrenaline evoked beta adrenergic dilatation of precapillary sphincters in skeletal muscle

By

JAN LUNDVALL AND JAHN HILLMAN

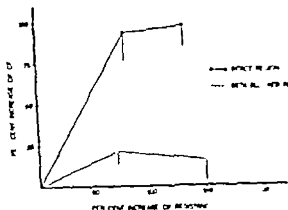
Blood-borne noradrenaline exerts a β-adrenergic dilator effect in the resistance vess skeletal muscle, but the inhibition of vascular tone is comparatively weak and is con by the simultaneous much stronger α-adrenergic constriction (e.g. Rapela and Green I Brick Hutchinson and Roddie 1967 Viveros, Garlick and Renkin 1968). The resi response evoked by activation of the sympathetic nerve fibres to skeletal musc includes such a seemingly insignificant β-adrenergic dilator component (Viveros et 1968) Recent investigations, however showed that this neurogenic β-dilator effect ca quite marked in the microvessels, especially in the "precapillary sphincters despite total muscle vascular resistance is only little influenced (Lundvall and Järhult 1974. The present study indicates that blood borne noradrenaline evokes a similar marked preferential β-adrenergic dilatation of the precapillary sphincters.

Methods

10 cats anesthetized with hloralose (50 mg/kg b.wt.) and urethane (100 mg/kg b.wt.) were and right lower leg muscles were isolated and the preparation was placed in a water-filled ple (38°C) t permit volumetric determination of the cap llary filtration coefficient (Mellander 1960, Lu and Järhult 1976) Arterial inflow pressure and venous outflow of blood were continuously Noradrenaline was administered by lose rterial infusion in dose of 1.0–3 0 μg/ml kg muscle b and after regional β-blockade. Each infusion period lasted 6 min with intervals of 8–10 min to p complete recovery of vascula tone in between the infusions. Propranolol was given close ar (600–800 μg/kg muscle) t block the β-adrenoceptors and concomitantly venous outflow of blood discarded to avoid systemic effects. Test experiments usi g the dextroisomere of propranolol showed the effect of the β-blocking agent to be reported was not related to an unspecific influence, such anesthetic ne

Results

Noradrenaline administration to the muscle vascular bed with intact β-adrenoceptors (tact region") invariably evoked a constriction of the resistance vessels, which was developed within 90 s and then maintained approximately constant during the infu period The steady state increase of regional resistance above control level ranged betw 50–200 % and was roughly related to the amount of infused noradrenaline. In the β-blo



1 Diagram showing the effect of noradrenaline infusion on CFC (capillary sphincter tone) in ... muscle before and after ...ckade. The data are classed with regard to the concomitant increase of vascular resistance and ... as mean value ± S.E. ... the number of observations.

...cle region the pattern of resistance response to noradrenaline was similar but the magnitude of the resistance increase was somewhat more pronounced for a given dose (25).

The capillary filtration coefficient (CFC), reflecting the number of patent capillaries and, hence, precapillary sphincter tone, was determined in the control state before each period of noradrenaline administration and after 2 3 min of infusion when the evoked resistance response had reached a steady state. Control CFC was quite stable in the individual animal and was unaltered by β-blockade. In the whole material control CFC varied between 0.011 and 0.017 ml/min 100 g mmHg, the mean value being 0.014 ± 0.001 (S.E.) ml/min 100 g mmHg (n 47). Noradrenaline administration to the intact muscle region always caused a dilatation of the precapillary sphincters as evidenced by a clearcut increase of the CFC. The CFC increment above control level ranged between 45 and 160 per cent and showed no consistent relation to the amount of infused noradrenaline. After β-blockade, the increase of CFC was much reduced or abolished and in several of the infusion experiments a decrease of the CFC was instead observed. Fig. 1 summarizes the CFC alterations during noradrenaline infusion (classed mean values ± S.E.), expressed in per cent of the control values prior to infusion and plotted against the concomitant per cent increase of regional vascular resistance (increase of resistance by 50–100 and 100–200 per cent, respectively). It can be seen that the mean increase of CFC in the intact muscle region was about 100 per cent at the moderate as well as at the more intense constriction of the resistance vessels. In the β-blocked muscle region there was also an increase of the CFC, on the average, but the increment was much smaller ($p < 0.001$) and amounted only to 19 and 16 per cent, respectively.

Comments

The significant effect of blood-borne noradrenaline on peripheral vascular functions is considered to be vasoconstriction caused by α-adrenoceptor activation. Vasodilatation due to activation of β-adrenoceptors has thus been regarded of little importance since, in the resistance vessels, the β-adrenergic inhibition of vascular tone is greatly overwhelmed by

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Blood-borne noradrenaline exerts a β-adrenergic dilator effect in the resistance skeletal muscle, but the inhibition of vascular tone is comparatively weak and b by the simultaneous much stronger α-adrenergic constriction (e g Rapela and Gr Brick, Hutchinson and Roddie 1967 Viveros, Garlick and Renkin 1968). The response evoked by activation of the sympathetic nerve fibres to skeletal m includes such a seemingly insignificant β-adrenergic dilator component (Vivei 1968) Recent investigations, however showed that this neurogenic β-dilator eff quite marked in the microvessels, especially in the "precapillary sphincters de total muscle vascular resistance is only little influenced (Lundvall and Järhult 19 The present study indicates that blood-borne noradrenaline evokes a similar m preferential β-adrenergic dilatation of the precapillary sphincters.

Methods

10 cats anesthetized with chloralose (50 mg/kg b wt.) and urethane (100 mg/kg b wt) ere right lower leg muscles were isolated nd the preparation was placed in water-filled pleth (38°C) to permit volumetric determination of the capillary filtration coefficient (Mellander 196 and Järhult 1976) Arterial inflow pressure and venous utflow of blood were continuously Noradrenaline was dministered by close rterial infusion in dose of 1.0–3.0 μg/min kg mu and after regional β-blockade Each infusion period lasted 6 min with intervals of 8–10 mi complete recovery of vascular tone l between the infusions. Propranolol was given clos (600–800 μg/kg muscle) to block the β-adrenoceptors and concomitantly venous outflow of discarded t void systemic effects. Test experiments using the dextroisomere of propranolol s the effect of the β-blocking agent to be reported was not related t an unspecific influence, anesthetic one.

Results

Noradrenaline administration to the muscle vascular bed with intact β-adrenocep tact region") invariably evoked a constriction of the resistance vessels, which developed within 90 s and then maintained approximately constant during the h period. The steady state increase of regional resistance above control level ranged b 50–200 % and was roughly related to the amount of infused lrenaline. In the β-

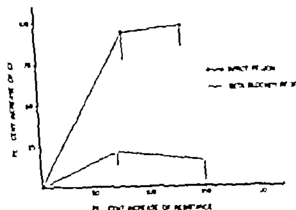


Diagram showing the effect ...radrenaline infusion on CFC ...pillary sphincter tone) in ...l muscle before and after ...ckade. The data are classed ...regard to the concomitant ...se of vascular resistance and ...ts mean value ± S.E. ...de the number of observations.

...de upon the pattern of resistance response to noradrenaline was similar but the ...gnitude of the resistance increase was somewhat more pronounced for a given dose ...25).

...he capillary filtration coefficient (CFC), reflecting the number of patent capillaries and, ...ce, precapillary sphincter tone, was determined in the control state before each period ...noradrenaline administration and after 2–3 min of infusion when the evoked resistance ...ponse had reached a steady state. Control CFC was quite stable in the individual ...mal and was unaltered by β-blockade. In the whole material control CFC varied ...ween 0.011 and 0.017 ml/min × 100 g × mmHg, the mean value being 0.014 ± 0.001 (S.E.) ...min × 100 g × mmHg (n = 47). Noradrenaline administration to the intact muscle region ...ays caused a dilatation of the precapillary sphincters as evidenced by a clearcut increase ...he CFC. The CFC increment above control level ranged between 45 and 160 per cent ...showed no consistent relation to the amount of infused noradrenaline. After β-blockade, ...increase of CFC was much reduced or abolished and in several of the infusion experi...nts a decrease of the CFC was instead observed. Fig. 1 summarizes the CFC alterations ...ring noradrenaline infusion (classed mean values ± S.E.), expressed in per cent of the ...ntrol values prior to infusion and plotted against the concomitant per cent increase of ...ional vascular resistance (increase of resistance by 50–100 and 100–200 per cent, ...pectively). It can be seen that the mean increase of CFC in the intact muscle region was ...out 100 per cent at the moderate as well as at the more intense constriction of the ...istance vessels. In the β-blocked muscle region there was also an increase of the CFC, ...the average, but the increment was much smaller (p < 0.001) and amounted only to 19 ...d 16 per cent, respectively.

Comments

...e significant effect of blood-borne noradrenaline on peripheral vascular functions is ...nsidered to be vasoconstriction caused by α-adrenoceptor activation. Vasodilatation due ...activation of β-adrenoceptors has thus been regarded of little importance since, in the ...resistance vessels, the β-adrenergic inhibition of vascular tone is greatly overwhelmed by

the α-adrenergic constriction (*cf* resistance data given above). The present study how- strongly indicates that β-adrenergic relaxation is the dominating effect of blood-b noradrenaline in the precapillary sphincters. In this vascular section, the β-adrer inhibitory influence evidently overrides the α-constriction that has been reported evoked by noradrenaline (Mellander 1966) Accumulation of "vasodilator metab" as a result of the blood flow reduction induced by noradrenaline may contribute to any α-adrenergic constriction of the precapillary sphincters and explain the sph relaxation observed also after β-blockade (Fig. 1)

The observed pronounced β-adrenergic dilatation of the precapillary sphincter response to noradrenaline infusion was fully developed at doses causing only a mod increase of vascular resistance (Fig. 1) Preliminary data from an extended series of in fact indicate that the β-adrenergic inhibition of sphincter tone is marked alread such small doses of noradrenaline that hardly affect total muscle vascular resistance further that the noradrenaline induced dilatation of the precapillary sphincters may be large as that evoked by adrenaline. In the intact organism, catecholamines released the adrenal glands might exert an important β-adrenergic control of transcapillary exch as a result of the inhibition of precapillary sphincter tone and the attendant increase the capillary surface area available for transcapillary exchange. The blood-borne c cholamines may in this respect act in concert with the previously described neuro β-adrenergic control of precapillary sphincter tone (Lundvall and Järhult 1976)

The study was supported by gra t ..10 from the Swedish Medical Research Council and by a grant the Medical Faculty of the U lversity of Lund, Sweden.

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m the Department of Pharmacology Leo Pharmaceutical Products, Ballerup, Denmark

Kidney volume expansion and prostaglandin release by bradykinin. The effect of indomethacin pretreatment

By

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Abstract

J B. *Kidney volume xpansion and prostaglandin release by bradykinin. The effect ndomethacin pretreatment* Acta physiol. scand. 1978. *102* 129–136.

hen bradykinin was infused into the left renal artery in anaesthetized dogs hich were loaded mannitol in saline to produce diuresis of approximately 1 ml/min. Bradykinin initially in- nal blood flow (70 %), kidney volume (20 %), subcapsular pressure (85), urine PG-excretion ncrease (80 .) and decreased urine osmolarity (20 %) Kidney volume, diuresis and urine os- remained equally changed during continuous kinin infusion, hile other parameters subsided he control levels. When renal blood flow was maintained constant during the infusion of brady- the PG-excretion increased (130), the glomerular filtration decreased (60 .) and the as ac ed by decreased kidney volume, diuresis/natriuresis ith unchanged urine osmolarity Indo- treatment (2.5 mg/kg) decreased renal blood flow (35 %) and inhibited urine PG-excretion. n after indomethacin showed effects hich ere very similar to those observed before indo- treatment. It is concluded that increased intratubular volume is main determinant of bradykinin ncrease of whole kidney volume. The accompanying increase of intrarenal pressure does not ap- contribute to the kinin induced PG-release. The vasodilation by bradykinin seems independent of prostaglandins. The diuresis/natriuresis and decreased urine osmolarity are probably of hemo- c origin.

dney kinins, bradykinin and kallidin (Hial *et al.* 1976), are potent renal vasodilators roduce natriuresis (Webster and Gilmore, 1964 Heidenreich *et al* 1964 Barraclough ulls 1965, Willis *et al.* 1969) and increase renal prostaglandin synthesis (McGiff *et al.* The kinins also increase kidney volume (Stürmer and Berde 1963), which will raise bcapsular tissue pressure due to the low distensibility of the kidney capsule (Hebert rbes 1971, Olsen 1977).

mannitol-diuretic dogs ureteral constriction and kidney compression, both of which dures increased the intrarenal pressure to 40 mmHg, have previously been shown to nce the urinary excretion of prostaglandin E-lik material (Olsen *et al.* 1976). Since this sts causal relationship between the intrarenal pressure and the rate of urinary PGE tion one object of the present study was to investigate whether a correlation between

the two parameters existed during renal intraarterial infusion of bradykinin with or without constriction of the renal artery In addition the possible effects of the prostaglandins released by bradykinin was studied by using indomethacin as a tool to inhibit renal prostaglandin synthesis.

Materials and methods

12 female mongrel dogs (12 25 kg) which had been fasted overnight, were used for the expts. They were kept on a standard diet (Doggy®) with free access to water

The dogs were anesthetized with pentobarbital sodium (30 mg/kg i.v) and intubated for spontaneous respiration. A Statham (P23Db) transducer was connected to a femoral artery to measure blood pressure and a catheter was placed in the femoral vein to permit subsequent infusion. The abdomen was opened by a midline incision. The left ureter was cannulated for urine collections and a flow probe was placed around the left renal artery for the measurement of renal blood flow (electromagnetic flow meter, Statham tron®)

A 25-gauge needle attached to a polyethylene catheter was introduced into the left renal artery distal to the probe and used for intrarenal infusions. Patency was ensured by a continuous infusion of saline at a rate of 0.2 ml/min. In 6 of the expts. a thread sling for manual constriction was also placed around the left renal artery to maintain renal blood flow constant during the kinin infusion. The left kidney was further prepared for the measurements of kidney volume (mercury strain gage, connected to a model 270 plethysmograph, Parks Elec. Lab) and subcapsular pressure (P 19 miniature transducer Kösingsberg Inc. Blood pressure, renal blood flow kidney volume and subcapsular pressure were continuously recorded on a Grass 7C polygraph At the end of the expt. the dog was killed by a lethal dose of pentobarbital. The subcapsular pressure recorded at this stage was used as zero value. The kidney was isolated, vessels were ligated. The plethysmograph was then calibrated by stepwise arterial infusions of saline.

When the continuously measured parameters had stabilized 15–30 min after surgery one group of dogs were loaded initially with 100 ml 10% mannitol in saline (10 ml/min) and 2 ml/min was infused throughout the expt When urine flow had stabilized 100 ng/kg/min bradykinin (Sandoz) was infused into the renal artery for 30 min, and urine was collected for periods of 15 min before, during and after infusion.

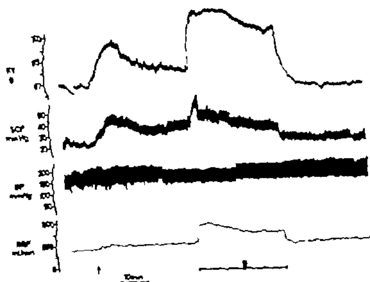
In a second group of 6 dogs the loading procedure was initiated immediately following completed surgery When urine flow had stabilized 100 ng/kg/min bradykinin was infused into the renal artery 12 15 min with and without renal artery constriction in random sequence. These expts. were continued by indomethacin (Dumex) administration (2.5 mg/kg i.v) followed by 100 ng/kg/min bradykinin i.r. 30 min later Urine was collected for 10 min before and during the infusions.

Analyses

Urine sodium was determined by flame photometry and creatinine by Jaffes reaction. Urine osmolality was measured with a semimicro osmometer (Knauer model M). Urinary excretion of prostaglandin-like material was measured by bioassay Urine was acidified (pH 3) with 5% formic acid and extracted with chloroform. The chloroform phase was partially evaporated and the residue chromatographed on silicic acid (0.5 g Biosil®). Non polar lipids were eluted with chloroform while prostaglandins were eluted with chloroform methanol (1 8). The PGE like activity was assayed on a rat fundus strip which is especially sensitive to prostaglandin E_2 (Splawinski *et al.* 1973 Crutchley and Piper 1975). The specificity of the assay procedure was further assured by the use of Krebs-Henseleit solution with a mixture of antagonists containing (in µg/ml) methysergide 0.2, propanolol 3.0, phenoxybenzamine 0.1 atropine 0.1 and phenhydramine 0 1 Recoveries of added prostaglandin E_2 (Unilever) were 85±2% (N=10) After silicic acid chromatography selected samples were applied on precoated silica plates for thin layer chromatography The plates were developed in the solvent system chloroform methanol:acetic acid (90:9:1 0 65). After zonal division the plates were scraped off into plastic tubes and it was assured that the majority (>90%) of the biologically (rat fundus strip) active material co-chromatographed with the PG standard.

Statistics

The results are presented as mean ± S.E. The significance of the changes was assessed by Student's t test on paired comparison after a logarithmic transformation.



The loading procedure as started at A, and 100 ng/kg/min bradykinin as infused into the renal at B. KV = kidney volume, SCP = subcapsular pressure, BP = femoral artery blood pressure and renal blood flow

Results

1 shows the effect of the loading procedure on the continuously registrated parameters representative expt. When diuresis was stable, (Table I) there were significant increases kidney volume (7%), subcapsular pressure (31%) and systemic mean blood pressure %). Renal blood flow did not change significantly. Fig. 1 shows the effect of 100 ng/kg/ bradykinin subsequently infused into the renal artery. Renal blood flow, kidney volume subcapsular pressure immediately rose to maximum levels 1–2 min after the infusion started. Renal blood flow and subcapsular pressure then fell towards control levels but there was no significant difference between the kidney volume expansion in the early phase of the kinin infusion compared with the final phase (Table II). Systemic blood pressure (Table I) remained unchanged during the infusions.

Bradykinin significantly increased urine volume, sodium and PG-excretions, and reduced urine osmolarity (Table II and III). The increase in sodium and PG-excretions was greater the first 15 min period of a sustained kinin infusion than in the second (Table II).

TABLE I. The effect of extracellular volume expansion on blood pressure and kidney parameters. Results are mean ± S.E. (N 6)

	Before expansion	During stabilized expansion	P
Kidney volume (g)	76 ± 5	81 ± 8	0.05
Subcapsular pressure (mmHg)	16 ± 3	21 ± 3	< 0.05
Renal blood flow (ml/min)	179 ± 30	177 ± 28	n.s.
Blood pressure (mmHg)	123 ± 6	141 ± 7	< 0.02

TABLE II The effect of bradykinin on kidney parameters during enhancement of renal blood flow ng/kg/min bradykinin was infused into the renal artery from 0 to 30 min. Results are mean (N = 6) Symbols in column represent comparisons between the two infusion periods (0–15 v.s 15–30 min). Otherwise the results have been compared with preinfusion levels (−15–0) n.s. = not significant = $p < 0.05$ = $p < 0.01$ = $p < 0.001$

min	÷15–0	0–15	15–30		0–[illegible]
Kidney volume (ml) g	81±8	97±8	96±8	n.s.	[illegible]
Subcapsular pressure mmHg	21±3	39±6	31±4		[illegible]
Renal blood flow ml/min	177±28	299±53	258±37		[illegible]
Diuresis ml/min	1 0±0 1	1.8±0.2	1.8±0.1	n.s.	[illegible]
Sodium excretion μeqv/min	31±5	103±29	70±18		[illegible]
Urine osmolarity mosm/l	622±39	487±23	444±39	n.s.	[illegible]
PG-excretion ng/min	2.4±0.8	12.3±4 9	7 6±2.5		[illegible]

No significant correlations were observed between kinin induced changes of renal bl[illegible] flow—subcapsular pressure (r = 0.24), subcapsular pressure—urine PG excretion (r [illegible] renal blood flow—urine osmolarity (r = 0.29), urine PG excretion—urine osmolarity (r [illegible] or renal blood flow—sodium excretion (r = 0.24) The levels of each of these param[illegible] after kinin infusion were significantly correlated with the control levels (Table IV).

TABLE III. The effect of bradykinin on kidney parameters during constant renal blood flow and indome[illegible] cin treatment. 100 ng/kg/min bradykinin was infused I to the renal artery and renal blood [illegible] is allowed to increase (II) or is maintained constant (III). At (V) bradykinin is infused [illegible] after the administration of 2.5 mg/kg indomethacin I. Results are mean ± S.E. (N [illegible] not significant, = $p < 0.05$ = $p < 0.01$ = $p < 0.001$

	Non-pretreatment			Statist. assess.		Indomethacin pretreatment			
	Control I	Kinin infusion				Control IV	Statist. assess. IV/I	Kinin infusion	
		RBF increase II	RBF constant III	III/I	III/II			V	St[illegible] VI
Subcapsular pressure mmHg	20±3	36±4	10±2			11±2		31±6	n.s.
Renal blood flow ml/min	155±22	64±36	170±31	n.s.		101±17		192±28	
Diuresis ml/ml	1 6±0.3	2.9±0.4	0.9±0.3			1.3±0.2	n.s.	2.6±0.3	n.s.
Sodium excretion μeqv/min	81± 6	245±56	35±24			45±17	n.s.	163±39	n.s.
Creatinine excretion μmol/min	1 7±0.4	2.0±0.5	0 7±0 2						
Urine osmolarity mosm/l	607±73	440±35	533±89	n.s.		550±71	n.s.	428±52	n.s.
PG-excretion ng/min	1.6±0.7	7.9±[illegible]3	6.9±2.1		n.s.	<		<8	

V Correlations between bradykinin induced changes of some kidney parameters and their control levels. Kinin induced changes of renal blood flow and subcapsular pressure represent mean levels observed during the first 10–15 min of infusion. (N = 12.)

ter	Correlation coefficient	Regression line	P
blood flow	0 95	y = 1 70x − 0 61	< 0 001
sular pressure	0 64	y 0 99x + 17 11	< 0 05
m	0.76	y = 1 18x + 0 89	< 0 02
n excretion	0 70	y = 156x 82.37	0 05
osmolarity	0 72	y 0.41 + 192.31	< 0 05
cretion	0 82	y 4 57x + 1.51	0 01

. 2 and Table III show the effect of 100 ng/kg/min bradykinin infused into the renal y during constant renal blood flow. The urine creatinine excretion decreased to approximately 40% of the control level and significant reductions of diuresis, sodium excretion and subcapsular pressure were observed. Further Table III shows that the arterial striction significantly changed the effects of the kinin infusion on all the parameters pt the urine prostaglandin excretion.

domethacin treatment (Table III, IV Fig. 2) significantly reduced renal blood flow pproximately 65% of control. This was paralleled by decreased subcapsular pressure. e prostaglandin excretion decreased below the range of sensitivity of the assay procedure ng/min). The changes of diuresis, sodium excretion or urine osmolarity were neither istent nor significant. 100 ng/kg/min bradykinin produced a diuretic/natriuretic response r indomethacin treatment which was not significantly different from that observed before omethacin treatment. The level of renal blood flow during kinin infusion after indomethacin treatment was, on the other hand, significantly lower than that observed before indomethacin treatment, but there was no significant difference between the increases in renal od flow produced by the kinin infusion before or after indomethacin administration.



. 2 100 ng/kg/min bradykinin as infused into the renal artery at A, B and D. Renal blood flow was maintained constant during A, and 2.5 mg/kg indomethacin was administered at C. BP = femoral artery ood pressure, SCP = subcapsular pressure and RBF = renal blood flow

Discussion

In the present expts. the renal intraarterial bradykinin infusion produced a marked r sustained expansion of the kidney volume. This was accompanied by an increased s capsular pressure (expansion pressure) which, however declined toward the control l during sustained enhancement of kidney volume, presumably as a result of chang capsular distensibility (Hebert and Arbus 1971 Olsen 1977) The most important effec kidney volume expansion will probably derive from the accompanying enhancement of t subcapsular pressure which reflects an increase of the interstitial pressure (Hebert and A 1971).

In similar experimental conditions urinary prostaglandin excretion has been found increase during enhancement of the intrarenal pressure (Olsen *et al* 1976). Yet, the sa increase in urine prostaglandin excretion by bradykinin in expts. where the subcaps pressure increased (renal blood flow increased) and those where the subcapsular pres decreased (renal blood flow maintained constant) indicates that changes of intrarenal p sure apparently is not an important factor in the kinin induced prostaglandin release. T results thus accord with the concept that bradykinin releases prostaglandins by a direct b chemical activation of an acyl hydrolase (phospholipase) (Kunze and Vogt 1971).

Renal i.a. infusion of bradykinin has previously been shown dose-related (5-500 ng min) to increase renal blood flow and urine electrolyte excretion without affecting the g merular filtration rate in pentobarbital anesthetized and non-loaded or saline loaded (Webster and Gilmore 1964 Heidenreich *et al* 1964 Barraclough and Mills 1965, W *et al* 1969). During constant renal blood flow bradykinin decreased urine creatinine ex tion which accords with the decreased inulin clearance (glomerular filtration rate) obser by Willis *et al* (1969)

The ability of bradykinin to increase sodium excretion and to decrease urine osmola is presumably related to the enhancement of renal blood flow since these effects disappea when renal blood flow was maintained constant. Noticeable was the observation that effect of bradykinin on kidney parameters (Table IV) including the urine prostagl excretion depended so much upon the control levels.

Exogenous prostaglandin E is usually a potent vasodepressor and natriuretic substa In the present expts. the level of renal blood flow during bradykinin infusion was lo after indomethacin pretreatment than before such treatment. This could suggest that p taglandins released by bradykinin amplified the vasoactivity of the peptide. This interpr tion was advanced by McGiff *et al* (1975) In isolated canine kidney these authors observed a decreased level of renal blood flow evoked by bradykinin, when the kidneys w pretreated with indomethacin. Indomethacin alone, however markedly reduced renal bl flow (Table III., McGiff *et al* 1975) The lower level of renal blood flow during bradyki infusion after indomethacin pretreatment might thus alternatively be explained by lowered preinfusion level of renal blood flow induced by indomethacin, since a hi significant relationship was found to exist between preinfusion and kinin infusion leve renal blood flow in non-pretreated expts. (Table IV).

One might speculate why indomethacin pretreatment in the present expts. failed

ancing effects of the prostaglandins released by bradykinin. Bradykinin and prostaglandin however share very similar effects on renal hemodynamic and excretory functions. The ked effect of bradykinin at the infusion dose used in the present expts. might perhaps ceal possible effects of intrarenally released prostaglandins. It might therefore be more ropriate to investigate the renal kinin-prostaglandin interrelationship in conditions ere intrarenally increased kinin activity mediates the prostaglandin release.

turner and Berde (1963) observed that kidney volume expansion accompanied the enacement of renal blood flow evoked by bolus kinin injections. In the present results 2 ervations suggest that the kinin induced enhancement of renal blood flow is not the only erminant of kidney volume changes. Thus kidney volume remained equally expanded ing sustained infusion of bradykinin, although the enhancement of renal blood flow nificantly decreased, and subcapsular pressure (kidney volume) decreased during the in infusion when renal blood flow was maintained constant. Kidney volume and urine however changed in parallel, which might imply that tubular olume is an important tor to determine whole kidney volume (Ostvik *et al.* 1971 Olsen 1977). The results are mplementary to those of Chinard *et al.* (1965). Using *in situ* dog kidneys these authors nonstrated that changes of renal blood flow were closer related to changes of the creatie distribution space than to changes of the vascular volume. Bradykinin has been shown increase capillary filtration and interstitial fluid (lymph) production in several vascular s (Haddy *et al.* 1970). The significance of this in relation to kidney volume expansion ms negligible, since kidney volume decreased during the kinin infusion when renal blood w was maintained constant.

Two other observations in the present study confirm that kidney volume may be related both changes of renal blood flow and/or diuresis. Thus, kidney volume and diuresis reased during the loading procedure although renal blood flow did not change signifintly while kidney volume and renal blood flow decreased after indomethacin treatment thout significant changes of urine flow

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A quantitative study of the insulin release induced by vagal stimulation in anesthetized cats

By

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Abstract

…ÄS-WALLENSTEN, K. and G. NILSSON. *A quantitative study of the insulin release induced by vagal stimulation in anesthetized cats.* Acta physiol. scand. 1978. *102*. 137–14…

…lin was released by vagal stimulation in anesthetized and eviscerated cats. The plasma insulin concentration and blood flow in the portal vein were determined concomitantly and the insulin output was calculated. Stimulation of either the right or the left cervical vagus released the same amount of insulin, whereas bilateral stimulation released twice as much. Following stimulation that depleted the "vagally-releasable pool" a recovery period of 15–20 min was needed before the same maximal output could be obtained again. With shorter interstimulatory periods the amounts of insulin released were reduced. When less than 2–3 000 impulses were applied during the stimulation period, the amount of insulin released per impulse was constant. Atropine (0.2–2 mg/kg) did not reduce the vagally-induced insulin release.

…dies in which the vagal nerves have been stimulated electrically indicate that such stimulation releases insulin (Kaneto et al., Frohman et al. 1967, Daniel and Henderson 1967). …n acute expts. on cats quantitative estimations of vagally-induced insulin output were …ently made by concomitantly recording the blood flow and the plasma insulin concentra…n the portal vein (Uvnäs-Wallensten and Nilsson, in press). The release mechanism …oved to be easily fatigued on stimulation with frequencies above 3–4 Hz. Irrespective of frequency or duration of stimulation, the release declined as soon as a total of 2–3 000 pulses had been applied. The existence of an insulin pool, available for immediate release, …d amounting to approximately 2% of the total pancreatic insulin store, was suggested. The present study was undertaken to obtain further quantitative information on vagally-…uced insulin release. These studies concern the effect of unilateral and bilateral vagal …mulation, the characteristics of the release mechanism as to duration, fatigue and re…very. In addition the relation between number of impulses applied and insulin release …ponse were investigated as well as the effect on insulin release by atropine.

Methods

…periments were performed on cats that had been fasted for 18 h. The cats were anesthetized with chlo…ose (50 mg/kg) and urethane (100 mg/kg). The intestine below the duodenum and the spleen was re-

TABLE I Amounts of insulin released following stimulations of either the right or the left vagus in the cats. R = right, L = left.

Exp. No	Number of impulses	Insulin released g	
1	1 500	410	R
	1 500	470	L
2	2 400	150	R
	2 400	190	L
3	2 400	1 540	R
	2 400	1 320	L
4	3 600	4 850	R
	7 200	4 890	R
	9 000	4 800	L

moved. The portal vein was ligated close to its bifurcation and the distal stump of the superior vein was cannulated. The blood draining from the stomach, the pancreas and the duodenum of the cat, passed through a drop-chamber unit and returned to the cat via a cannulated femoral vein.

The cervical vagal nerves were stimulated uni- or bilaterally with a Grass stimulator (S8) giving pulses of 7–10 V 2 3 ms duration and 1–20 Hz. A more detailed description of the surgical procedure has been presented elsewhere (Uvnäs-Wallensten and Nilsson, in press). Plasma insulin concentration was measured by radio-immunoassay as described by Nilsson and Uvnäs-Wallensten (1974). The insulin output was calculated by multiplying portal insulin concentration and plasma flow. The vagally-induced insulin release was determined by subtracting the basal insulin release from the total insulin output obtained during stimulation of the nerves.

Results

Unilateral and bilateral stimulation

Stimulation of either the right or left vagal nerve (n = 4) released similar amounts of insulin provided that the same number of impulses were delivered (Table I).

When bilateral and unilateral stimulations of the same frequency and duration were performed in a cat (n = 4), bilateral stimulation released about twice as much insulin into portal blood as did unilateral stimulation (Fig. 1).

As shown in Fig. 2, a maximal insulin response could be induced by stimulation of

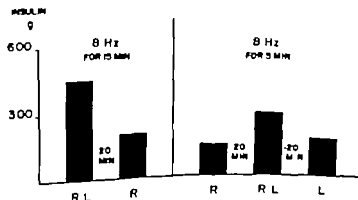
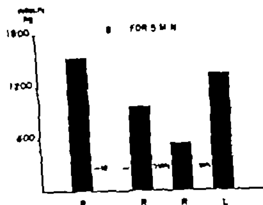


Fig. 1 2 expts. demonstrating release of insulin following unilateral and bilateral vagal stimulation (8 Hz for 5 and 15 min). Note the additive effect of bilateral stimulation.



Expt. demonstrating the fatigue of in- release following repeated supramaximal stimulations of either the right or the ... (5 Hz for 5 min 2 400 imp) ... ying interstimulatory periods. Note stimulation of the left nerve could re- full amounts of insulin immediately af- ... stimulation of the right nerve.

...agal nerve immediately following supramaximal stimulation of the right vagal nerve, when the release mechanism of the right nerve was fatigued (n = 3).

...ue of release mechanism

... a supramaximal ... agal stimulation (> 2 3 000 impulses) a subsequent supramaximal ...ulation released smaller amounts of insulin, unless the release mechanism was allowed ...covery period of 15–20 min (n = 4). The shorter the interstimulatory period, the smaller amount of insulin released by subsequent stimulations. The fatigue phenomenon is ...trated in Fig. 2.

...in release per impulse

... to the rapid fatigue of the insulin release mechanism, the output of insulin per impulse ... difficult to estimate. However on submaximal stimulation (less than 3 000 impulses), ...nstant relationship was found between the amount of insulin released and the number ...mpulses applied, regardless of the frequency or duration of stimulation (Fig. 3 a, b, c ... Table II).

...ect of atropine on vagally-stimulated insulin release

... identical unilateral vagal stimulations (5 Hz for 10 min) were performed in 11 different ... The interstimulatory period was 30 min. In 6 expts. atropine (0.2–2 mg/kg) was ad- ...nistered i. ... 10 min before the second stimulation. The insulin release following the second ...ulation was expressed as a percentage of that caused by the first stimulation. In the ...trol expts ... the insulin release following the second stimulation was 102 % of that after ... first (86–124). Atropine did not reduce the insulin output within the dosage range ...ed. On an average, the insulin release caused by the second stimulation was 105 % of ... induced by the first (Table III).

Discussion

...a previous paper (Uvnäs-Wallensten and Nilsson 1975) the existence of an insulin pool ..., depletable by vagal stimulation, was suggested. The background to this pro-

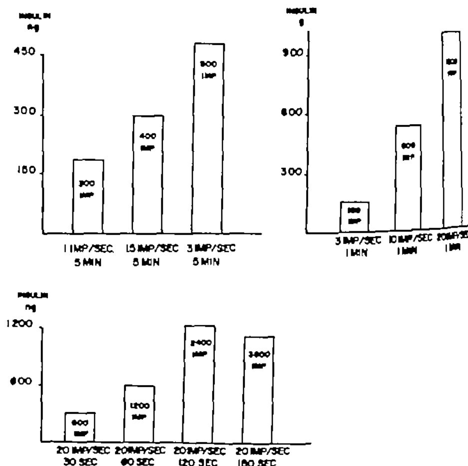


Fig. 3 a, b, c. 3 expts. demonstrating that the amount of insulin released per impulse is fairly co following vagal stimulation with less than 2 3 000 impulses. Either the frequency (a and b) or the tion of the stimulation ( ) was varied. The insulin/impulse ratio declined when more than 3000 i were applied (c) reflecting the f tigue of the release mechanism. Interval between stimulations 20 Table II).

posal was the observation that the insulin release mechanism proved to be easily fa following vagal stimulation Due to the long recovery period required (15–20 min), i considered unlikely that a cholinergic neurotransmission failure was responsible for fatigue phenomenon.

The results of the present study suggest that unilateral supramaximal stimulation of the right and the left vagi causes depletion of such an insulin pool, and that stimulat either of the nerves releases the same amount of insulin. Bilateral stimulation yields insulin than does unilateral stimulations, the output being approximately doubled unilateral release mechanisms seem to operate independently of each other since the output in response to a supramaximal stimulation of one vagal nerve is maximal, when the contralateral release mechanism has not yet recovered from a previous stimul

The explanation of the additive effect of bilateral vagal stimulation and the indepe behaviour of the vagal release mechanism is not clear The two vagal nerves might inne separate beta-cell populations. If this is the case, more insulin will be released on bil

II Insulin release on vagal stimulation with different frequencies or durations showing the output of insulin/impulse. Intervals between stimulations = 20 min (see Fig. 3).

Frequency Hz	Duration	Number of impulses	Insulin release ng	ng/imp.
1	300	300	200	0.7
1.5	300	450	310	0.7
3	300	900	520	0.6
3	60	180	160	0.9
10	60	600	530	0.9
20	60	1 200	1 000	0.8
20	30	600	310	0.5
20	60	1 200	600	0.5
20	120	2 400	1 [illegible]40	0.5
20	180	3 600	1 100	0.3

ulation, because more beta-cells are reached by the vagal impulses. Alternatively each cell might have a dual innervation, unilateral stimulation releasing only part of the in pool, accessible for vagal release. A sustained release of insulin, corresponding to ate of refilling of the "pool" should be expected to occur when vagal stimulation is nued after depletion of the pool. Such a continuous insulin secretion has not been rved. Therefore, it cannot be excluded that electrical vagal stimulation not only pro- s the release of insulin from the beta-cells, but also activates a mechanism inhibiting in release.

the same cat the insulin release per impulse following repeated submaximal stimulation 3 000 impulses) was found to be constant. This fact makes the cat preparation well d for testing substances which may influence vagally-stimulated insulin release. Atro- even in very high doses, did not inhibit the vagally-induced release of insulin. This may n that the vagal stimulation was not mediated by acetylcholine or that the acetylcholine tive receptors are not muscarinic.

III. Two successive vagal stimulations were performed at 5 Hz for 10 min in 11 cats. In cats number 1–6, atropine in doses ranging from 0.2–2 mg/kg was given 10 min before the second stimulation. The insulin output induced by the second stimulation is expressed as percentage of the preceding control stimulation. The interstimulatory period was 30 min.

No.	Atropine mg/kg	Insulin release induced by the second stimulation expressed as percentage of that induced by the first (control) stimulation
	0.2	130
	0.2	120
	0.5	70
	0.5	80
	1	110
	2	120
		105 (70–130)
	—	102 (96–124)

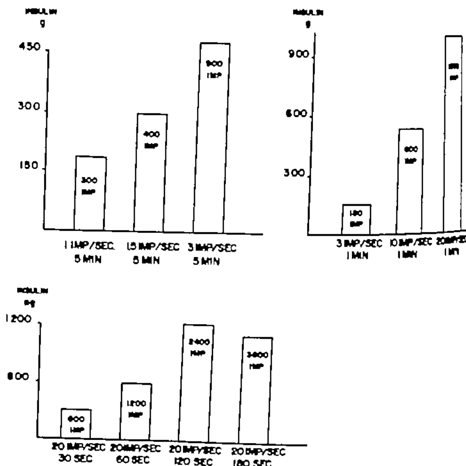


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Indirect vascular actions of (Gln⁴)-neurotensin in canine adipose tissue

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Received 16 June 1977

Abstract

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...vasoconstrictor action of the tridecapeptide (Gln⁴)-neurotensin has been studied in subcutaneous ...se tissue in the inguinal region of anesthetized dogs. Close intra-arterial infusion of (Gln⁴)-neurotensin 30–120 pmol · kg⁻¹ b.wt. · min⁻¹ elicited similar vasoconstrictions in the adipose tissue on the ...ion side and on the contralateral side. This suggests that (Gln⁴)-neurotensin must enter the general ...rculation before it can elicit vasoconstriction. Removal of parts of the gastrointestinal tract did not ...ge the vasoconstrictor response. Thus, there is no indication of release of vasoactive substances from ...gastrointestinal tract by (Gln⁴)-neurotensin. Infusion into the portal vein elicited the same vasoconstriction in adipose tissue as the same dose administered i... It is suggested that the vasoconstrictor action in adipose tissue is not caused by (Gln⁴)-neurotensin *per se*. Instead, vasoactive substance(s) ... be formed from (Gln⁴)-neurotensin.

...rotensin is a tridecapeptide which has been isolated from bovine hypothalamus ...raway and Leeman 1973). The amino acid sequence has been established and the syn...s described (Carraway and Leeman 1975 a, 1975 b). Recently Folkers *et al.* (1976) ...gested that the acid analog (Gln⁴)-neurotensin rather than neurotensin may be the ...urally occurring peptide. In dogs, neurotensin and (Gln⁴)-neurotensin infused i.v. ... pmol · kg⁻¹ · min⁻¹ or more) have pronounced effects on certain functions in the ...trointestinal tract. Thus, they inhibit the gastric motor activity (Andersson *et al.* 1977) ...d the gastric acid secretion (Andersson *et al.* 1976).

The neurotensins also have vasoconstrictor action in some vascular beds, including ...nervated adipose tissue (Rosell *et al.* 1976). The vasoconstriction is delayed in onset, ...curring about 5 min after the beginning of i.v. infusion. Therefore, it was suggested ...at this effect and possibly other actions of neurotensins may be mediated by a neurotensin ...tabolite or by the release of some vasoactive substances(s) (Rosell *et al.* 1976). In the ...sent investigation these possibilities as far as the vasoconstriction in adipose tissue is ...cerned have been analyzed experimentally.

Frohman *et al* (1967) reported that the atropine blocks the insulin secretion b' vagal stimulation in the dog. It is difficult to evaluate their results since inhibition w and since they did not determine the effluent blood flow from the pancreas whi have fluctuated.

The ability of atropine (0.2 mg/kg) to inhibit the insulin release caused by sham f in dogs (Nilsson and Uvnäs-Wallensten 1974) may appear inconsistent with our results. However when high doses of quarternary anticholinergic drugs, which do n the blood-brain barrier were given instead, the insulin release in response to sham f was not affected at all (Uvnäs-Wallensten and Andersson, to be published). This probably blocks central neuronal pathways, which are involved in the induction sham feeding response.

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Indirect vascular actions of (Gln^4)-neurotensin in canine adipose tissue

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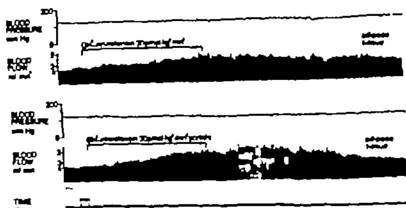


Fig. 2 Experimental record illustrating blood pressure and blood flow in acutely denervated subcutaneous adipose tissue. (Gln⁴)-neurotensin, 30 pmol kg^{-1} min^{-1} was infused intravenously (upper record) [into] the portal vein (lower record)

dominal viscera. The reference pattern was unaffected by splenectomy (2 expts.), ncreatectomy (3 expts.), removal of duodenum, jejunum, ileum or colon individually. However in those expts. where the whole small intestine was removed the responses were diminished although the latency and vasoconstrictor pattern was unchanged.

Infusion of (Gln⁴)-neurotensin into the portal vein. (Gln⁴)-neurotensin infused into the portal vein in doses ranging between 30–120 pmol kg^{-1} min^{-1} produced vasoconstriction in adipose tissue which was of the same magnitude as the vasoconstriction following i.v. administration. Moreover the latency and the duration of the vasoconstriction were similar irrespective of the routes of administration (Fig. 2). The vasoconstriction, expressed as the area under the peripheral resistance time curve, was not significantly different from that following the same dose administered intravenously (Student's t-test for paired observations, n = 6) (Fig. 3)

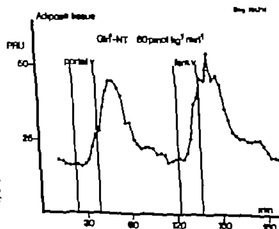


Fig. 3 Changes in vascular resistance in subcutaneous adipose tissue in one dog following infusion of (Gln⁴)-neurotensin, 60 pmol kg^{-1} min^{-1} intravenously or

Methods

The experiments were performed on 19 female mongrel dogs weighing 9–25 kg, anesthetized [illegible] pentobarbital, 30 mg/kg with supplement as necessary Tracheotomy was performed, and the dogs [illegible] ventilated with a Braun Melsungen model 74052 respirator To prevent coagulation, heparin (2500 IE [illegible]) was administered. Subcutaneous adipose tissue in the left and the right inguinal regions was isolated [illegible] skin and other surrounding tissues (Rosell 1966). This procedure provides two almost identical [illegible] tissue preparations, each supplied by one artery one vein and one mixed nerve. The tissues [illegible] denervated. Blood flow was measured using a silicon-filled drop recorder inserted into the arterial [illegible] of the tissue. Systemic blood pressure was measured in a carotid artery with a Statham P23Ac [illegible] and recorded together with blood flow on a Grass model 7B polygraph. Vasoconstriction [illegible] in peripheral resistance units (PRU mmHg × ml⁻ min 100 g^{-1}). To obtain a measure of the [illegible] vasoconstrictor effect of infused drugs in adipose tissue the area under the peripheral resistance [illegible] was calculated using the trapezoidal rule.

Drugs were administered by close intra-arterial infusions via a side arm in the arterial line just [illegible] to the drop counter Infusions into the portal vein were made via a polyethylene cannula inserted through [illegible] mesenteric vein. (Gln^4)-neurotensin (M W 1674) was dissolved in saline.

Results

Adipose tissue blood flow Infusion of (Gln)-neurotensin intraarterially (30-120 pmol kg⁻ × min) to the inguinal subcutaneous adipose tissue induced vasoconstriction after latency of about 5 min. As is evident from Fig. 1 i.a. infusion of (Gln^4)-neurotensin on one side induced similar vasoconstrictions on the infusion side and contralateral side as far as latency degree and duration of vasoconstriction are concerned These results indicate that (Gln^4)-neurotensin must enter the general circulation before inducing vasoconstriction in adipose tissue.

Partial removal of the gastrointestinal tract One possible mechanism for the vasoconstrictor action of (Gln)-neurotensin is that the polypeptide releases vasoactive substances In order to examine this possibility abdominal organs were extirpated in acute expts. and the effects of these procedures on the vasoconstriction in subcutaneous adipose tissue were investigated. (Gln)-neurotensin was infused i.v before and after the extirpations of vari[illegible]

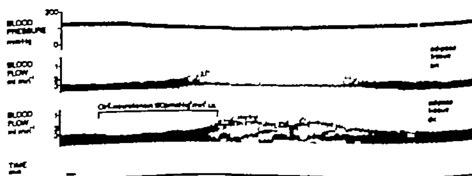


Fig. 1 Experimental record illustrating blood pressure and blood flow in acutely denervated subcutaneous adipose tissue. Blood flow was measured in both the left (upper record) and the right (lower record) adipose tissue preparations. (Gln^4)-neurotensin, 120 pmol kg^{-1} min^{-1} was infused into the arte[illegible] the right adipose tissue

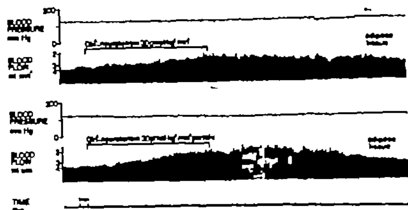


Fig. 2 Experimental record illustrating blood pressure and blood flow in acutely denervated subcutaneous adipose tissue. (Gln[4])-neurotensin, 30 pmol kg⁻¹ min⁻¹ was infused intravenously (upper record) or into the portal vein (lower record)

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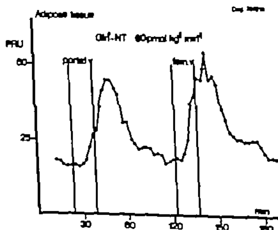


Fig. 3 Changes in vascular resistance in subcutaneous adipose tissue in one dog showing infusion of (Gln[4])-neurotensin, 60 pmol kg⁻¹ min⁻¹ intravenously or into the portal vein

Discussion

Previously reported experiments have shown that in doses above 18 pmol × kg⁻¹ ... i.v. both neurotensin and (Gln)-neurotensin elicit a delayed vasoconstriction in denerv... canine subcutaneous adipose tissue and to a lesser degree in the skin and small intes... Due to the long latency of about 5 min following i.v. administration, it was suggested t... the vasoconstriction in adipose tissue was indirectly mediated. The vasoconstriction was ... blocked by adrenergic α-receptor blocking agents or by bilateral ligation of the renal pedic... Thus, the vasoconstriction does not seem to be mediated by catecholamines or by eff... on the renin-angiotensin system or other renal mechanisms. Furthermore, the vasoco... strictor action does not seem to be mediated via the central nervous system since infus... of neurotensins into the vertebral artery produced smaller effects than similar dos... administered i.v. (Rosell *et al* 1976)

The present expts. were aimed at further testing the hypothesis of an indirect action ... (Gln⁴)-neurotensin. Apart from a slight initial vasodilatation, close intra-arterial infusi... of (Gln⁴)-neurotensin to the inguinal adipose tissue did not induce any changes in periphe... resistance that could be ascribed to a direct action of the peptide. Instead i.a. infusions ... (Gln⁴)-neurotensin on one side produced similar vasoconstrictions on the infusion side a... contralateral side. This result indicates that (Gln)-neurotensin must enter the gene... circulation before it can elicit vasoconstriction in the adipose tissue. Therefore it is possib... that (Gln)-nebrotensin releases some vasoconstrictor substance or alternatively is metab... lized to one or more peptides with vasoconstrictor properties. Both these alternatives hav... been considered in the present series of expts. The results of expts. in which abdomin... organs or parts of the gastrointestinal canal were extirpated did not indicate any relea... The reduction of the vasoconstrictor response following extirpation of large parts of th... gastrointestinal tract may be a consequence of the decrease in the portal blood flow and thus an altered distribution of the infused (Gln)-nebrotensin.

The finding that infusion of (Gln)-neurotensin infused into the portal vein elicits th... same vasoconstriction as the same dose administered i.v. indicates that the peptide is no... inactivated in the liver. Intead, (Gln⁴)-neurotensin may be activated to vasoactive substance... by *e.g.* cleavage of the neurotensin molecule to partial sequences. Presumably such a con... version does not take place in the liver since one would then have expected a greate... vasoconstriction after infusion into the portal vein than after i.v. infusion. The reaso... being that following i.v. infusion only about 25% of the total dose will be distributed t... the liver whereas 100% of the dose will pass the liver after intraportal administratio... Whether some of the partial sequences of neurotensin have vasoconstrictor properties ... not known. Carraway and Leeman (1975 c) have tested partial sequences of neurotensin o... several biological parameters and found that COOH-terminal sequences of 5 or mor... amino acids in length have full intrinsic activity. Whether formation of these peptides ma... occur in plasma or in some other tissue remains to be investigated.

Apart from their vasoactivity in small doses in adipose tissue and the gastrointestina... tract, neurotensins are potent inhibitors of gastric antral pressure motility and gastric aci... secretion stimulated by pentagastrin or a test meal (Andersson *et al* 1976, 197...

l 1976). At higher doses, neurotensin produce elevation of blood glucose (Carraway ners and Leeman 1973, Rosell et al 1976). Whether or not some or all of these effects directly mediated by neurotensin or are due to metabolites remains to be determined.

rt investigation was appropriately supported by grants from the Swedish Medical Research Council D, Åke Wibergs Stiftelse and from the Robert A. Welch Foundation. Heparin was generously supplied AB Vitrum, Sweden

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The effects of cholera toxin on intramural blood flow distribution and capillary hydraulic conductivity in the cat small intestine

By

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Abstract

CEDGÅRD S D A. HALLBÄCK, M JODAL, O LUNDGREN and S REDFORS. *The effects of cholera toxin on intramural blood flow distribution and capillary hydraulic conductivity in the cat small intestine* Acta physiol. scand 1978 *102* 148 158

Blood flow distribution t the mucosa-submucosa and to the muscularis in the cat small intestine investigated with a ^{85}Kr elimination technique before and after exposing the intestinal mucosa for ... to cholera enterotoxin. In all experiments the toxin induced n intestinal secretion. Concomitantly, ... intestinal blood flow was ncreased t a level 50 per cent above control 3 h after exposure. This ... tion reflected a doubling of mean blood flow in the mucosa-submucosa while muscularis blood ... remained unchanged In another series of experiments the effect of cholera toxin on intestinal capillary hydraulic conductivity was investigated by determining the cap llary filtration coefficient (CFC) A ... increase in CFC was noted during the 3 h observation period but this was not more pronounced ... would ha e been expected from the concomitant asodilatation. It is concluded that hemodynamic ... in the intestinal mucosa may be one of the several f ctors that probably are invol ed in the pathogenesis of cholera

The cause of the profuse fluid loss in cholera is still disputed Two fundamentally different mechanisms have been proposed (*cf* Field 1974) The currently most accepted hypothesis infers that the produced fluid is the result of an increased "active secretion" and/or a decreased "active" absorption induced by the cellular action of the enterotoxin produced by the *Vibrio cholerae* organism This hypothesis is mainly based on *in vitro* studies in which the involved hydrostatic, osmotic and electrochemical forces ... be strictly controlled.

The other hypothesis implies that the driving force for the intestinal fluid production in cholera is the capillary hydrostatic pressure. This hypothesis was originally proposed

The term "secretion denotes i this paper a net water transport into the intestinal lumen regardless of the underlying mechanisms.

rchow (1879) based on histopathological investigations of patients that had died of cholera. ese studies suggested that the intestinal villi became denuded when exposed to the olera vibrion. However intestinal biopsies taken from living cholera patients demon-rated only small changes of intestinal morphology (see e.g. Gangarosa et al 1960) thout any signs of an intestinal villous destruction. Recent reports suggest, however at the cholera exotoxin may increase the hydraulic conductivity of capillaries and/or of intestinal epithelium even in the absence of histological changes (see e.g. Yeardley et al .73).

In order to investigate the possible involvement of circulatory parameters in the patho-nesis of the fluid losses in cholera the present study was undertaken and two types of idies were performed on the cat small bowel. In one type of experiments total blood flow d intramural flow distribution in the gut were investigated before and after exposing the testinal epithelium to cholera toxin. In a second type of experiments the effect of cholera xin on the hydraulic conductivity of the intestinal capillaries was studied.

Methods

Operative procedures and determination of total intestinal blood flow

experiments [illegible] are performed on cats anesthetized [illegible] [illegible] th chloralose (50 mg/kg b wt.) after induction th ether. The animals [illegible] re deprived of food for 24 h and had no obvious signs of intestinal infection. e operative procedures used [illegible] ere similar to those of previous studies of intestinal circulation per-rmed in this laboratory (Folkow et al 1963, Kampp et al. 1968). After [illegible] tracheotomy the abdomen [illegible] as ened in the midline and the greater omentum and the spleen were extirpated. The experiments [illegible] ere rformed on [illegible] 15–20 cm long isolated segment from the jejunum, the remainder of the gastrointestinal act being extirpated. The lumen of the segments was carefully rinsed [illegible] th body-warm saline.

After heparinizing the animal (3–5 mg/kg b. [illegible] t.) blood pressure [illegible] as measured by [illegible] pressure transducer [illegible] (Statham P23 AC) in the femoral artery. The superior mesenteric vein, draining the intestinal segment d its lymph nodes, was cannulated and connected to an optical drop recorder—ordinate [illegible] rter unit, cording on [illegible] Grass polygraph. The venous blood was returned to the animal via the right jugular vein, the nous outflow pressure being kept around 10 mm Hg. The sympathetic nervous control [illegible] as eliminated cutting the splanchnic nerves bilaterally.

Determination of blood flo[illegible] distribution

ood flow in the mucosa–submucosa and in the muscularis was determined with [illegible] ^{85}Kr wash-out method, ing [illegible] simplified modification of the technique originally described by Kampp et al. (1968). The radio-tive isotope ^{85}Kr [illegible] as purchased as gas from Radiochemical Centre, Amersham, England, and was solved in [illegible] 0.9 per cent saline solution. The body-warm solution (0.5 to 1.0 ml) [illegible] as injected intra-arterially single bolus during 5 to 10 [illegible] sec [illegible] cannula in [illegible] marginal intestinal artery. T[illegible] record muscularis blood w the disappearance of β-radioactivity from the muscularis was recorded by means of [illegible] Geiger Müller [illegible] indow counting tube (Philips No 18504, diameter of window 9 mm), enclosed in lead, and placed the antimesenteric border of the small intestine. The G–M tube was coupled to [illegible] rate meter (Packard [illegible] Oceanus series 410 A), operating an ink writer. When counts exceeded 5 000 per min, correction [illegible] as ade for [illegible] coincidence loss using [illegible] value of 100 μs for the dead time of the G–M tube. T[illegible] prevent diffusion the gas from tissue to air the intestinal surface and the mesentery was covered by Mylar®. Contamination the air [illegible] ith ^{85}Kr was reduced by continuous suction around the tracheal cannula.

Counts per min were plotted vs. time on semilogarithmic paper. I[illegible] most instances [illegible] straight line was tained but in [illegible] few experiments arched curves were recorded. In these instances the straight tail part the curve was used, the initial fast elimination rate in all probability reflecting the tracer [illegible] sh out from intestinal mucosa (cf. Kampp et al. 1968). Intestinal blood flow (F) in ml per min and 100 g muscularis sue was calculated according to Kety (1951) as

$$F = k \lambda 100 \qquad (I)$$

where k denotes the rate constant of the monoexponential decay and λ the blood-tissue partition coeffic for ^{85}Kr The latter constant was set to 1 The k value was determined according to

$$k = \frac{\ln 2}{t_{1/2}}$$ (

where $t_{1/2}$ denotes the half time of disappearance in min as calculated from the wash-out curve

Blood flow in the mucosa–submucosa was calculated from the total intestinal blood flow muscle blood flow and the relative weights of the mucosa–submucosa. The last mentioned weights were der mined for each experiment by dissecting the muscularis free from the mucosa–submucosa and weigh separately

C. *Recording of intestinal net water transport*

In all ^{85}Kr wash-out experiments except one, the rate of intestinal net water transport was follow continuously with the technique described by Jodal *et al* (1975). Briefly the lumen of the isolated intes segment was perfused at a constant rate (1 ml/min) with a solution of known composition (see below) closed perfusion system with a reservoir large enough to prevent recirculation. Changes in the reservoir volume was continuously recorded by a volume transducer coupled to a Grass polygraph and connect to the perfusion system *via* a T tube. When no intestinal motility occurred the changes in the perfu volume reflected net water transport across the intestinal epithelium. In these experiments atrop (1 mg/kg b.wt.) was given to minimize motility

D *Plethysmographic technique*

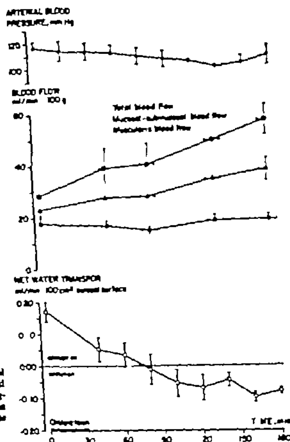
To be able to estimate the hydraulic conductivity of the intestinal capillaries (*i e* their capillary filtrat coefficient CFC) the small intestines and its lymph nodes were enclosed in a triangular perspex plethym graph according to the technique originally described by Folkow *et al* (1963). The plethysmograph w filled with a physiological saline solution or Tyrode solution and connected to a volume transduc recording on Grass polygraph. The temperature of the plethysmograph was maintained at 37 C, continuously controlled by thermocouple thermometer (Electrolab, Copenhagen). Polyethylene tube inserted into the ends of the intestinal segment were fitted through openings in the plethysmogr making it possible to perfuse the lumen of the intestinal segment inside the plethysmograph. Tempor increasing the venous outflow pressure to a known extent (usually 10 cm H_2O) evoked a characteristic volume increase in 2 phases. As earlier demonstrated (Mellander 1960, Kjellmer 1965 Wallentin 1966 first rapid change in volume is caused by a vascular distension, mainly of the veins, while the slower increa in volume, "the filtration slope" reflects an outward transcapillary filtration caused by the rise in m capillary hydrostatic pressure.

To be able to calculate the capillary filtration coefficient (CFC) it is necessary to correlate the net outward filtration to the induced change in mean capillary hydrostatic pressure. The extent to which increase of venous pressure is propagated to the capillary level depends on the relation between pre- postcapillary resistances of the vascular bed (Pappenheimer 1953). To estimate this ratio in the pres study a mean capillary hydrostatic pressure of 20 mm Hg was assumed Folkow *et al* (1963) and Hög and Lundgren (1972) discussed at length the error of CFC determination if the true hydrostatic capill pressure was 10 mm Hg above or below the assumed one. It was found that the error thus introduced amounted to only 10–15 per cent, as long as the arterial blood pressure remained within the normal ra (around 100 mm Hg).

E. *Experimental procedures*

After completing the operative procedures the animal were allowed to rest for 45–60 min. At the end this period control measurements of the different parameters studied were made. 50–200 mg per 10 intestine of a crude cholera toxin (freeze dried culture filtrate; NIH lot 001) dissolved in 5–10 ml physiolog saline was then introduced into the intestinal lumen. The toxin was kindly supplied to us by dr Holmgren, Department of Medical Microbiology University of Göteborg. After 30 min exposure intestinal lumen was rinsed with 50–100 ml bodywarm saline. The animal was studied for another during which all animals included in this series developed signs of fluid loss from the intestinal epithel

In 4 control expts. the cholera toxin solution was heated to 56°C for 45–60 min to inactivate the tox This solution was then introduced into the bowel lumen as described above. In these expts. total intest blood flow and net water absorption rate were monitored.



1 Arterial blood pressure, intestinal
d flow (total and intramural) and
water transport in an intestinal seg-
t before and following 30 min
sure to cholera toxin. Bars denote
SE 7

Solution

e lumen of the intestinal segments were perfused with modified Krebs-Henseleit solution containing
mol/l: Na 122, KCl 4.7, KH_2PO_4 1.2, $MgCl_2 \cdot 6H_2O$ 1.2; $CaCl_2$ 2.5, mannitol 30. The osmolality of
solution ranged between 305 and 315 mOsm/kg H_2O. If necessary the osmolality of the solution was
justed to a value within 10 mOsm/kg H_2O of that of plasma by addition of appropriate amounts of
nnitol.

Results

a. Blood flow and flow distribution

ntestinal blood flow and flow distribution were studied with the [85]Kr elimination technique before and after a 30 min exposure of cholera toxin. The results obtained are illustrated in Fig. 1 (total and intramural blood flow: net water transport) and Fig. 2 (blood flow distribution). Time 0 denotes the start of the cholera toxin incubation period. Blood flows are expressed per 100 g of each individual tissue.

It is evident from Fig. 1 that the cholera toxin produced the expected change of direction of net transport of water. Approximately 60 min after the exposure to the toxin, a net fluid

where k denotes the rate constant of the monoexponential decay and λ the blood-tissue partition coeff. for ^{85}Kr. The latter constant was set to 1. The k value was determined according to

$$k = \frac{\ln 2}{t_{1/2}}$$

where $t_{1/2}$ denotes the half time of disappearance in min as calculated from the wash-out curve.

Blood flow in the mucosa-submucosa was calculated from the total intestinal blood flow, muscle blood flow and the relative weights of the mucosa-submucosa. The last mentioned weights were determined for each experiment by dissecting the muscularis free from the mucosa-submucosa and weighing separately.

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In 4 control expts. the cholera toxin solution was heated to 56°C for 45–60 min to inactivate the toxin. This solution was then introduced into the bowel lumen as described above. In these expts. total intestinal blood flow and net water absorption rate were monitored.

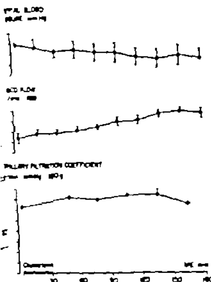


Fig. 3

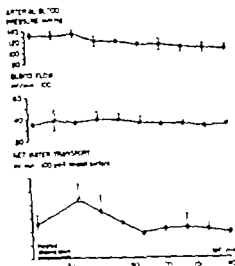


Fig. 4

. 3. The effects of cholera toxin on arterial blood pressure, intestinal blood flow and intestinal capillary ration coefficient. Bars denote ± S E. =7

. 4. The effects of heated cholera toxin on arterial blood pressure, total intestinal blood flow and nal net ater transport. Bars indicate ± S E. =4.

s submucosa constituted 10 per cent of the intestinal weight (Jodal and Lundgren 1970). cond, blood flows in the muscularis and in the submucosa were assumed to be identical. is Table reveals more clearly than Fig. 1 and 2 the redistribution occurring in the gut in sponse to cholera toxin.

The hydraulic conductivity of intestinal capillaries

e hydraulic conductivity of the intestinal capillaries was estimated from determinations the capillary filtration coefficient (CFC). Such measurements were performed before d after exposing the intestinal segment to the cholera toxin. 3–5 measurements were ade in the control period and the average value of these was taken as the control value. g. 3 summarizes the results obtained in 7 expts., *including the observed changes in arterial* ood pressure and venous outflow. Although total intestinal blood flow increased to the me extent as illustrated in Fig. 1 CFC exhibited only a slight tendency of increase. Comring the control measurements with the last measurements in each expt. an increase of the draulic conductivity was observed n all expts. making the difference statistically nificant (p 0.02, Wilcoxon's matched-pairs signed-ranks test).

Control experiments

4 expts. the cholera toxin solution was heated to 56°C for 45–60 min prior to intestinal cubation. After such a treatment no intestinal vasodilatation or intestinal fluid loss were duced by the cholera toxin solution (Fig. 4).

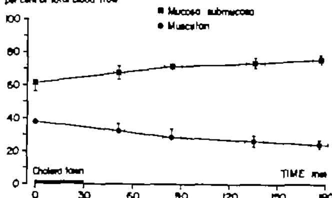


Fig. 2. Changes in blood flow distrib— within the intestinal wall following min exposure to cholera toxin. The are based on the blood flow values Fig. 1 Bars indicate ± S.E. n 7.

loss into the intestinal lumen was recorded Concomitantly total intestinal blood fl increased. The present analyses revealed that this vasodilatation was confined to the muco submucosa In this wall layer blood flow doubled during the period of observation wh blood flow in the muscularis remained largely unchanged. This response pattern was al reflected in the observed changes in blood flow distribution With increasing total intesti blood flow an increasing fraction of total intestinal blood flow was diverted to the muco submucosa (Fig 2)

In 4 expts. blood flow in the mesenteric lymph nodes was studied before and 3 h aft exposing an intestinal segment to cholera toxin Blood flow in the lymph nodes was de determined after gently occluding the vessels supplying the intestine during short perio An increased lymph node flow was recorded after cholera toxin but it was of approximat the same relative magnitude as the one observed for total blood flow Hence, the increase total blood flow illustrated in Fig. 1 cannot be explained by an exaggerated vasodilatat confined only to the lymph nodes.

A further analysis of the blood flow redistribution occurring after exposure to chol toxin is presented in Table I In this Table the blood flow and flow distribution to ea individual layer has been calculated making two assumptions. First, it was assumed th

TABLE I Blood flow and flow distribution before ("co trol") and 3 h after exposing the small bowel cholera toxi ("cholera"). Mean ± S E. n = 7

	Control	Cholera
Total blood flow ml/min 100 g	.3 ± 2	36 ± 4
Regional blood flow ml/min × 100 g		
mucosa	3 ± 4	65 ± 7
submucosa	18 ±	17 ±
muscularis	18 ± 2	17 ±
Blood flow distribution, per cent		
mucosa	54 ± 6	71 ± 3
submucosa	8 ± 1	5 ± 1
muscularis	38 ± 5	24 ± 3

ond, a number of histological observations seem to indicate an increased capillary meability in cholera. Thus, increases of capillary permeability to colloidal iron-dextran w above 150 000; Keusch *et al.* 1967), ferritin (m.w. around 346 000: Dalldorf *et al.* ...) and horseradish peroxidase (m.w. 40 000: Yardley *et al.* 1973). Furthermore, most -opathological studies indicate the presence of a tissue edema in cholera located to the cosa and the submucosa (Gangarosa *et al.* 1960) and often wide "dilated" capillaries the villi (Yardley *et al.* 1973).

However in the present study no experimental support for a substantially increased draulic conductivity of the intestinal capillaries in cholera was obtained, to judge from only slightly increased capillary filtration coefficient (CFC) in the face of a fairly marked cosal vasodilatation. Inducing a similar blood flow increase by a vasodilator drug, with effect on capillary permeability clearly increased CFC (Folkow *et al.* 1963). The tracers d in the studies cited above were all of high molecular weight, passing the capillary lls through "leaks" (Grotte 1956) and/or by pinocytosis (Carter *et al.* 1974). The fluid nsfer through "leaks" and by pinocytosis is in all probability contributing very little to over capillary hydraulic conductivity (Grotte 1956). The present results may therefore be plained by assuming that the cholera toxin only increased capillary permeability to ge molecules by increasing the size of occasional capillary "leaks" and/or augment nocytosis while the conventional "pores" of the fenestrated mucosal capillaries were not luenced by the toxin. An alternative explanation may be that a change of capillary rmeability was confined only to a small portion of all intestinal capillaries.

In this context it should be underlined, however, that the absence of any direct effect of olera toxin (m.w. 84 000: Finkelstein and LoSpalluto 1972) on capillary permeability in is study may be due to the relative impermeability of the intestinal epithelium to such ge polar solutes as the toxin. The increase of vascular permeability demonstrated in the ... experiments cited above, may therefore not be relevant with regard to the situation the intestinal wall.

Mean capillary hydrostatic pressure in cholera. The tissue edema often reported in the oleraic intestinal wall (see above) may not necessarily reflect an increased capillary rmeability but may be the result of an increased mean capillary hydrostatic pressure ...). The results of the present study make it possible to calculate the increase of capillary essure necessary to produce the rate of secretion observed in this study about 3 h after the posure to the cholera toxin. Assuming that the capillary hydraulic conductivity of the pillaries in the muscularis and in the submucosa is the same as in skeletal muscle, i.e. ...10 of that of the whole intestine (*cf.* Cobbold *et al.* 1963; Folkow *et al.* 1963), the hydraulic nductivity of the intestinal mucosal capillaries can be calculated. In this study one then rives at a mucosal capillary filtration coefficient of 0.15 ml/min · mmHg · 100 g mucosal sue. Mean absorption rate in the control situation amounted to 1.11 ml/min · 100 g ucosa and mean secretory rate at 3 h after cholera exposure was about 0.52 ml/min · 100 g ucosal tissue. Hence, an increase of P of 11 mmHg ((1.11 + 0.52)/0.15) would produce e secretory rate observed in the feline gut of this study after cholera toxin exposure, ssuming that all the filtered fluid reached the intestinal lumen. This is not probable for veral reasons, one being that filtered fluid is also transported towards the "inside" of the

Discussion

The present study has revealed that the incubation of cholera toxin in the kit produced within 1–2 h a secretory state, as also reported by Finck and Katz (1972) Dlugolecka (1974) Quantitatively the secretory rate was the same as the highest inte absorptive rate observed in the cat. In man that would be equivalent to a secretion of 400 ml/h Furthermore, the net secretion of fluid induced by the cholera toxi accompanied by an increase of total intestinal blood flow An analysis of the intra blood flow distribution revealed that the vasodilatation occurred exclusively in the mu submucosa were blood flow was doubled 3 h after exposing the intestine to the toxin hydraulic conductivity of the intestinal capillaries was, however only slightly increa the toxin despite the induced, quite substantial mucosal vasodilatation.

Intestinal blood flow in cholera With regard to the effects of cholera toxin on inte hemodynamics, two earlier studies have been published by Carpenter *et al.* (1968 Norris and Summer (1974) Carpenter and coworkers reported on an average i increase of superior mesenteric artery blood flow in the dog 1 h after the administrati the cholera toxin to the stomach, *i.e* a vasodilatation of the same magnitude as rep here (Fig. 1 and 3)

Norris and Summer (1974) on the other hand, neither recorded any blood flow c upon exposing the intestine to the cholera toxin, nor any significant change of intra blood flow distribution. In their investigation a technique was used that in princip similar to the present one. However Norris and Summer made a compartment an of the wash-out of γ-activity recorded after i.a injection of ¹³³Xe, as was done by K *et al* (1968) Our reason for not using this approach was that one cannot, without extensive control experiments, assume that the different components of a multifac desaturation curve reflect the same biological events in two such different experim conditions as the normal and the choleraic intestines. As no such control studies wer formed, the results by Norris and Summer are difficult to evaluate.

The mechanism underlying the cholera-induced vasodilatation is not known. It improbable that products from the villous cells are responsible for the hyperemia the great majority of vascular smooth muscles controlling mucosal blood flow are lo in the vessels situated in the deeper mucosal parts. Recently however a high concent of vasoactive intestinal peptide (VIP) was demonstrated in the lumen of choleraic int (Bloom *et al* 1977) VIP is a "candidate hormone" in the gut (Grossman 1974) that al been proposed to be a neurotransmittor This may suggest that the cholera toxin in the release of certain hormones in the crypts that may exert a local effect on the vessels and/or the toxin elicits an intramural nervous reflex.

Capillary permeability in cholera Two types of experiments seem to suggest that c toxin increase capillary permeability which, in turn, may contribute to the pathogen cholera. First, injecting the toxin into the skin of guinea pigs or rabbits (Craig 1965 F stein *et al* 1966) produced a local tissue edema indicative of an effect on vascular pe bility This effect could not be dissociated from the effects of the cholera toxin even using the most purified preparations of the toxin (Finkelstein and Lo Spalluto

...re absorption. However Lee and Silverberg (1972) and Strombeck (1972) have ...orted results which seem incompatible with this view by demonstrating that intestines ...n dog and rat, which during *in vivo* conditions secreted fluid after exposure to cholera ...n, ...ere capable of fluid absorption during *in vitro* conditions. Thus, most probably the ...stinal fluid loss in cholera is the result of several mechanisms, increased capillary ...ation being one. Furthermore, we are currently investigating the effects of the cholera ...n on the countercurrent multiplication of sodium chloride in the villi. The villous ...erosmolality created by this mechanism is probably of great importance for the intestinal ...orption of water (Jodal *et al.* 1978). Preliminary experiments suggest that exposing the ...e gut to cholera toxin markedly diminishes tissue osmolality in the villi, thus further ...lerlining the multifactorial background to the cholera secretion.

...search ...as supported by grants from the Swedish Medical Research Council (14X 2855), from the ...dish Society for Medical Sciences, from Harald and Greta Jeanssons Fund, from Wilhelm and Martina ...idgrens Fund, from Magnus Bergvalls Stiftelse and from the Faculty of Medicine, University of ...eborg.

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mucosa (*i.e.* the villous core and the lymphatics) and another being that all mu- capillaries may not be adjacent to the lining epithelium. These calculations clearly sug- that the hydraulic conductivity of the intestinal mucosal capillaries is high enough to account for large filtration rates at comparatively small changes in P_c.

The question then arises: did any hemodynamic changes occur in the gut after expo- to cholera toxin which may produce an increased P_c? One may then recall that blood f- doubled in the mucosa, probably mainly due to a vasodilatation at the precapillary le- Hence, a marked increase of P may have been induced. Unfortunately with the pre- method it is not possible to determine the pre- to postcapillary resistance ratios. Howe- it has been demonstrated in skeletal muscle that a doubling of blood flow by exe- augments tissue volume at a rate corresponding to an increase of P of about 5 mm (Kjellmer 1964).

The discussion above has been carried out assuming that the intestinal epithelium d- not offer any resistance to transepithelial fluid transport. However, this assumption probably not true since most epithelia are considered to have a hydraulic conduct- well below that of capillaries. In fact, for a long time most epithelia were considered perfectly tight, only allowing fluid transport of water through cellular "pores". How- evidence has lately accumulated that epithelia may be quite leaky, particularly when pres- is applied to the "tissue" side, at least during *in vitro* conditions. For example, Hakim- Lifson (1969) obtained a value of the epithelial hydraulic conductivity which was 10 t- larger than that of the capillary permeability when expressed per unit area. A comparat- high leakiness has also been observed in similar experimental condition in other epit- (for ref. see House 1974). Moreover, according to some authors, epithelial permeabilit- increased in cholera (see *e.g.* Love 1969).

To summarize, earlier reports and the present study have provided experimental sup- for the view that increased capillary permeability (mainly for large molecules) and increased mean capillary pressure in the mucosa may contribute to the intestinal fluid l- seen in cholera, although it cannot be considered to be proven. In reviews these propo- are dismissed with reference to the results obtained by Carpenter *et al.* (1969). This stud- the only one in which a direct attempt has been made to investigate the possible invo- ment of capillary forces in cholera. Dogs were infected with cholera vibrios and during t- acute phase of the disease arterial blood pressure was lowered for periods of 90 min. Fo- lowering arterial blood pressure to 50 mm Hg or below did not reduce the rate of chole- fluid loss. However, these results do not exclude the involvement of capillary forces i- cholera for at least two reasons. First, even pronounced changes of arterial blood pres- affects P very little by proper adjustment of the pre- to postcapillary resistance ratio, d- to the myogenic autoregulatory capacity of the intestinal vascular bed (Haglund a- Lundgren 1972, Gore and Bohlen 1975). Second, the mere lowering of arterial blood pres- to 50 mm Hg or below for 90 min may in itself produce a net fluid loss into the intesti- lumen also in most normal animals (Haglund and Lundgren 1972). Such a fluid loss ca- be distinguished from the cholera diarrhea.

As indicated in the introduction the currently most accepted hypothesis infers that the f- loss in cholera reflects an exaggerated "active" intestinal secretion and/or an inhibition

...ove absorption. However Lee and Silverberg (1972) and Strombeck (197?) have ...rted results which seem incompatible with this view by demonstrating that intestines ...l dog and rat, which during *in vitro* conditions secreted fluid after exposure to cholera ...n, ere capable of fluid absorption during *in vitro* conditions. Thus, most probably the ...tinal fluid loss in cholera is the result of several mechanisms, increased capillary ...ation being one. Furthermore, we are currently investigating the effects of the cholera ...n on the countercurrent multiplication of sodium chloride in the villi. The villous ...erosmolality created by this mechanism is probably of great importance for the intestinal ...orption of water (Jodal *et al.* 1978). Preliminary experiments suggest that exposing the ...e gut to cholera toxin markedly diminishes tissue osmolality in the villi, thus further ...erlining the multifactorial background to the cholera secretion.

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Aspects on diamine oxidase activity and its determination

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Abstract

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...mogenates of guinea-pig liver and human placenta, tissues known to be rich in diamine oxidase, were ...ated with ^{14}C-putrescine and the metabolites formed were determined. I the incubate of guinea-pig ... the major metabolites were GABA and some unidentified compound(s), Δ -pyrroline and $^{14}CO_2$ were ...obtained. In both the maternal and fetal parts of human placenta, radioactive GABA and the un...fied compound(s) as well as Δ -pyrroline were found. The results indicate that GABA is an important ...mediate in putrescine metabolism. Determination of the amount of Δ^1-pyrroline is thus not ell suited ...measure of the diamine oxidase activity in tissues.

...words: Diamine oxidase activity, putrescine metabolism, GABA, Δ -pyrroline

...mine oxidase (EC 1.4.3.6) catalyses the oxidation of diamines such as histamine, putres... and cadaverine, yielding in the process an aminoaldehyde. The enzyme was thought ...ttack principally histamine and for that reason was named histaminase. It was later ...wn that putrescine and cadaverine are the preferred substrates (for ref. see Zeller 1965 ...dsley *et al.* 1970).

...is well established that when putrescine is incubated in the presence of purified diamine ...ase γ-aminobutyraldehyde is formed which almost quantitatively is transformed into ...cyclic compound Δ -pyrroline. The in vitro measurement of diamine oxidase activity ...ording to Okuyama and Kobayashi (1961) is in reality based upon the determination of ...Δ -pyrroline formed from added ^{14}C-putrescine to the incubate. The Δ -pyrroline is ...racted into a toluene liquid scintillation mixture for assay of the radioactivity. This ...hod is widely used, mostly for measurement of diamine oxidase activity in plasma. The ...ty is also used in the determination of diamine oxidase activity in tissues where the ...hod has not been well characterized.

...t has been shown that injection of ^{14}C-putrescine was followed by the expiring of large ...ounts of radioactive CO_2 within a couple of hours and γ-aminobutyric acid (GABA) was ...nd to be an intermediate in the catabolism of putrescine to carbon dioxide (Seiler and

Eichentopf 1975 Henningsson and Rosengren 1976) The catabolism of ¹⁴C-putrescine radioactive GABA and CO_2 was blocked by aminoguanidine, a specific diamine oxidase inhibitor

The aim of the present study was to determine the nature of the metabolic products formed from putrescine via the diamine oxidase pathway on incubating tissues in vitro and to develop another approach to the assay of diamine oxidase activity in tissues taking into consideration the main products formed in the incubate, thus applying our in vivo findings of putrescine catabolism on in vitro studies. A preliminary report of this work has been presented elsewhere (Andersson *et al* 1977)

Methods

Adult female guinea-pigs, weight 830–950 g, were used. They were fed a standard pellet diet and tap water *ad libitum*. The animals were stunned and exsanguinated whereafter pieces from the liver lobes were removed immediately The tissue was cooled on ice, minced with scissors, weighed and gently homogenized in a Dounce type homogenizer (25 strokes with the pestle) in 4 volumes of 0.1 M cold sodium phosphate buffer pH 7.2 containing 0.2 % (w/v) glucose. The homogenate was used as enzyme source

Human placenta was obtained at term and was divided into its maternal and fetal parts. The maternal part was taken from the outermost layer and the fetal part from a layer at depth of about 1 cm from the surface. Homogenate was prepared in the same way as above.

Incubation procedure The incubation mixture contained homogenate corresponding to 70, 80 or 100 mg of tissue, 1 4- ¹⁴C putrescine (final conc. 10^{-4} M, sp act. 0.1–5 mCi/mmol) and the same buffer as used for homogenization, the total finally made up to a volume of 1.5 ml. The mixture was incubated for different times at 37°C. Blanks were provided by preincubation with 100 µl aminoguanidine hemisulphate (final conc. 2 10^{-4} M). The incubation was stopped by tipping 0.7 ml 2 M hydrochloric acid (samples subjected to thin-layer electrophoresis) or 0.7 ml 12.6% (w/v) sulfosalicylic acid (samples subjected to automated liquid chromatography) into the incubate from a side arm of the incubation vessel. A further 45 min shaking followed for complete absorption of expelled $^{14}CO_2$ (see below).

Determination of radioactive substances resulting from catabolism of ¹⁴C-putrescine The following putrescine metabolites were determined CO_2, GABA, Δ^1-pyrroline and, in addition, some unknown compound(s). Each of these products (with the exception of $^{14}CO_2$) was measured in two different ways. Some incubates were used for assays by automated liquid chromatography Parallel incubates were divided into two parts, where one was used for extraction into toluene, the other for thin-layer electrophoresis. Duplicates were performed of all determinations with the exception for assays carried out with automated liquid chromatography Thin-layer electrophoresis and automated liquid chromatography were also used for determination of the amount of unmetabolized labelled putrescine at the end of incubation.

1 Expelled $^{14}CO_2$ was trapped on 10 25 mm piece of No 005 Munktell's filter paper prepared with 100 µl hydroxide of Hyamine 10-X. After termination of the incubation the filter paper was dropped into 8 ml of Bray scintillation mixture (Bray 1960) and the radioactivity was measured in a liquid scintillation spectrometer (Packard TRI-CARB, 2002).

2 Chromatographic separation of GABA was carried out using an automatic amino acid analyzer (BIOCAL 3201). Application of liquid chromatography in diamine research has been described by ... *et al* (1970) The procedure as carried out in our laboratory is as follows. After centrifugation and adjustment of pH to 2.0–2.5 with NaOH the incubate was filtered through a filter with a pore size of 0.2 µm (... corp., Bedford, Mass. 01730). The radioactive compounds of the incubate were separated at 60°C on a column of Durrum DC 6A. In separating amino acids and amines, four buffers with different pH and amounts of sodium citrate and sodium chloride were pumped through the column in sequence at flow rate of 80 ml/h for different elution times. Buff A: 0.20 M Na pH 3.90, 44 min Buff B 0.35 M Na pH ... 44 min Buff C 0.90 M Na pH 6.20, 47 min Buff D 2.80 M Na pH 5.90, 40 min. After passing the column the stream was split according to Lou (1973). One half proceeded to a fraction collector by means of which 2 ml fractions could be collected throughout the analysis. Of these fractions an aliquot of ... was added to 3 ml Instagel (Packard) scintillation solution and the radioactivity was measured. The other half of the column eluate was mixed with ninhydrin and peaks were ... ninh...

as in the regular way. The final identification of the compounds in relevant fractions of eluates from amino acid analyzer has been described (Hesterberg and Rosengren 1976).

addition radioactive GABA as estimated by thin-layer electrophoresis. Aliquots of the incubate centrifuged at 80 g for 20 min. Of the supernatant 250 μl as added to 5 μl of standard carrier solution (1 μg unlabelled GABA and 1 μg unlabelled putrescine in 5 μl) and 2.5 ml ethanol completed the precipitation of proteins. The samples were stored at 18°C until electrophoresis was performed. Next, mixture as centrifuged, the supernatant as evaporated to dryness, the residue as resolved in 25 μl 0% ethanol and quantitatively applied to thin-layer plate (TLC plates silica gel 60, Merck). The thin-layer electrophoresis which separated the radioactive compounds was performed at 20 V/cm for 70 min and at 3°C using pyridine-acetate buffer pH 4.8 (Fischer and Dörfel 1957). The plate as dried and the relevant fractions were removed in zones. The silica gel powder was transferred to vials containing 10 ml dilution solution (5.5 g Permablend III, Packard, in one litre of toluene) and the radioactivity as measured.

The procedure employed for determination of Δ^1-pyrroline as the method devised by Okuyama and Kobayashi (1961). Kusche et al. (1973) have given detailed description of the procedure. Aliquots of the incubate were mixed with saturated $NaHCO_3$ and NaOH solution to obtain pH of 9.5 ± 0.1. T ml of sample was transferred to test tube containing 4 ml cold toluene. The test tube, equipped with glass stopper, as vigorously shaken for 4 min, centrifuged at 900 g for min after which the aqueous phase frozen at −20 to −30°C in bath of ethanol and solid carbon dioxide. Four ml of the upper toluene phase was transferred to vials containing 4 ml scintillation solution (11 g Permablend III Packard, in one of toluene). The radioactivity was measured.

Determination of radioactive Δ-pyrroline was also carried out by the automatic amino acid analyzer described above. In identifying Δ^1-pyrroline, standard test solution was prepared by incubating ^{14}C-putrescine with purified diamine oxidase (hog kidney, Grade II, Sigma).

Hitherto unidentified radioactive compound(s) appeared in the samples and as determined by either of the two methods, i.e. the automated liquid chromatography or the thin-layer electrophoresis.

Results

Initial experiments it was shown that on incubating ^{14}C-putrescine with crude liver homogenate $^{14}CO_2$ as well as ^{14}C-Δ-pyrroline were formed while at incubation with the supernatant of the liver homogenate only pyrroline was formed (Table I). These expts. also showed that the catabolism of putrescine was considerably higher in the homogenate than in the supernatant. The results encouraged the search for intermediate metabolites of radioactive putrescine. In these expts. homogenate of guinea-pig liver was incubated with ^{14}C-putrescine 1 h. The putrescine metabolites, as well as the residue of added putrescine, were determined in either of two systems. The results are seen in Table II. Except for the unknown compound(s), a good agreement was obtained by the methods used. The discrepancy regarding the unknown compound( ) is the subject of further investigation. Using the automated liquid chromatographic method a split-stream technique is necessary to determine radioactive compounds, a technique which is rather expensive and laborious. For that reason we henceforth in this study refer to results obtained by the toluene extraction of pyrroline and by thin-layer electrophoresis of the other putrescine derivatives.

From Table II it appears that an inverse relation existed between on the one hand the amount of unmetabolized putrescine and, on the other the amount of GABA and the unknown compound(s), i.e. the more substrate used up the more GABA and unknown compound(s) were formed. Regarding pyrroline no such relation was found.

Experiments were performed to demonstrate the relationship between the rate of formation of the different putrescine metabolites and the amount of tissue in the incubate. Aliquots

Eichentopf 1975 Henningsson and Rosengren 1976) The catabolism of ¹⁴C-putrescine radioactive GABA and CO was blocked by aminoguanidine, a specific diamine oxidase inhibitor

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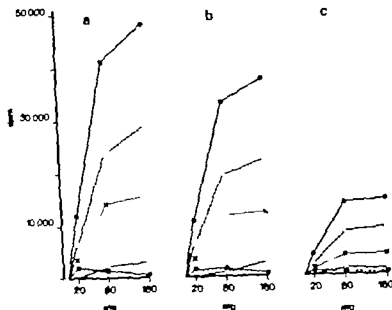
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Determination of radioactive substances resulting from catabolism of ¹⁴C-putrescine The following putrescine metabolites were determined CO_2, GABA Δ^1-pyrroline and, in addition, some unknown compound( ). Each of these products (with the exception of ¹⁴CO_2) was measured in t o different ... Some incubates were used for assays by automated liquid chromatography Parallel incubates were divided into two parts, where one was used for extraction int toluene, the other for thin-layer electrophoresis. Duplicates were performed of all determinations w th the exception for assays carried out with automated liquid chromatography Thin-layer electrophoresis and a tomated liquid chromatography ... for determination of the amou t of unmetabolized labelled putrescine at the end of incubation

1 Expelled ¹⁴CO_2 was trapped on 10 25 mm piece of No. 005 M ktell's filter paper prepared ... 100 µl hydroxide of Hyamine 10-X. After termination of the incubation the filter paper was dropped into 8 ml of Bray sci tillation mixture (Bray 1960) and the radioactivity was measured in a liquid scintillation spectrometer (Packard TRI-CARB 200..).

2. Chromatographic separation of GABA was carried out using an utomatic amino acid analyzer (LKB BIOCAL 3201). Application of liquid chromatography in diamine research has been described by ... *et al.* (1970). The procedure as carried out in our laboratory is as follows. After centrifugation and adjustment of pH to 2.0–2.5 with NaOH the incubate was filtered through a filter with pore size of 0 .2 µm (M... corp., Bedford, Mass. 01730). The radioactive compound of the incubate were separated at 60°C ... column of Durrum DC 6A. I separati g amino acid and amines, four buffers w th different pH and ... amounts of sodium citrat and sodium chloride were pumped through the column i sequence at a flow of 80 ml/h for different lution times Buff A: 0.20 M Na pH 3.50, 44 min Buff B 0.35 M N pH ... 44 min Buff. C. 0.90 M N pH 6.20 47 min Buff D 2.80 M N pH 5 90, 40 min. After passing the column the stream was plit according to Lou (1973) One half proceeded t fraction collector by means of which 2 ml fractions could be collected throughout the analysis. Of these fractions an liquot of ... was added to 3 ml Instagel (Packard) scintillation solution and the radioactivity was measured The other half of the column eluat was mixed with ninhydrin od peaks were p by ninh



1. Rate of formation of different metabolites from ^{14}C-putrescine in guinea-pig liver as related to the ount of tissue. The metabolites are CO_2 (○—○), Δ^1-pyrroline (●—●), GABA (○—○) and unknown mpound(s) ( ). The total amount of these metabolites is also given (●—●).

t not with 160 mg, this applicable to both the expts. with high and low oxidative deamina-
n.

The relationship between the incubation time and amount of products formed was also died (Fig. 2). The tissue amount in the samples was 20 mg in 2 expts. and 80 mg in two ers. GABA and the unknown compound(s) were again the major metabolites and the oduction of them was linear up to at least 1 h. With 80 mg of tissue there was a linear ation between the amount of $^{14}CO_2$ produced and the incubation time. With 20 mg of sue the production of $^{14}CO_2$ also rose linearly with increasing incubation time, but it was gligibly small. The time curve for Δ^1-pyrroline showed a progressively increasing course to 120 min in samples with 20 mg tissue. However in samples with 80 mg tissue the ount of Δ^1-pyrroline increased up to 60 min whereafter there was a decline in the formation Δ-pyrroline. The total amount of putrescine metabolites found plotted against time lowed a straight line up to 2 h in expts. with 20 mg tissue in the incubate. With 80 mg the rve was linear to only 60 min.

The oxidative deamination of ^{14}C-putrescine on incubating human placenta was also udied. 80 mg of tissue was incubated for 30 and 90 min. The degradation of ^{14}C-putrescine as approximately 10 times higher in the maternal part than in the fetal one. In both parts dioactive GABA and the unknown compound(s) were formed as well as Δ-pyrroline. rmation of $^{14}CO_2$ could also be recorded. In contrast to the expts. with guinea-pig liver -pyrroline was the major putrescine derivative. In the maternal part the amount of pyrro-

TABLE I Formation (nmol/g b) of radioactive Δ -pyrroline and CO_2 from ^{14}C-putrescine in homogenate (H) and supernatant (S) of guinea-pig liver in 3 experiments. The sum of the two products is given.

Δ^1-pyrroline		CO		Δ^1-pyrroline plus CO_2	
H	S	H	S	H	S
84	77	410	0.255	490	78
51	30	450	0.52	500	31
61	24.2	350	7 6	410	32

of homogenate corresponding to 20 80 and 160 mg of liver from the same animal were incubated for 1 h. The following products of ^{14}C-putrescine were noted CO_2, Δ^1-pyrroline, GABA and the unknown compound(s). The major metabolites formed were GABA and unidentified compound(s) (Fig. 1) In the three experiments referred to in Fig. 1 there was a linear relationship between tissue amount and rate of formation of these two metabolites up to 80 mg tissue per sample. With 160 mg of tissue the curve was flattening in all 3 experiments. As to $^{14}CO_2$, the production of this putrescine metabolite was low and an approximately linear relation to the tissue amount was found this is in contrast to Δ^1-pyrroline, the production of which was highest in the samples with low tissue amount. In Fig. 1 the total amount of the observed putrescine metabolites is also plotted. In 2 of the 3 expts. the oxidative deamination was high the sum of the products constituting 21 and 18% of the ^{14}C-putrescine substrate in samples of 20 mg, and 83 and 64%, respectively in samples of 160 mg (Fig. 1a and 1 b). The third liver had a lower enzyme activity the products constituting 7% in samples with homogenate of 20 mg tissue and 24% with 160 mg (Fig. 1c) The curve representing the sum of putrescine metabolites is approximately linear with the tissue amount of 20 and 80

TABLE II. Comparison of different methods used for the assay of metabolic products [Δ^1-pyrroline, GABA and an unknown compound( )] of ^{14}C-putrescine and the residue of putrescine after incubation of ^{14}C putrescine (58 000 dpm/sample) with liver homogenate. The methods used were automated liquid chromatography (ALC), toluene extraction (TE) and thin-layer electrophoresis (TLE). The figures are dpm/sample.

	Δ -pyrroline		GABA		Unknown compound( )		Putrescine	
	ALC	TE	ALC	TLE	ALC	TLE	ALC	TLE
	1 460	950	4 000	4 500	3 200	330	45 000	47 000
	248	410	8 900	15 600	5 300	5 900	—	29 300
	1 630	1 990	5 000	6 400	3 500	3 500	38 000	45 000
	1 460	1 350	27 100	23 400	20 400	14 100	5 300	8 500
	810	540	31 000	28 800	23 100	15 500	3 000	5 900
	1 410	1 370	6 700	5 900	5 600	3 300	48 000	46 000
	1 610	1 430	18 900	19 100	20 700	11 500	17 700	19 700
	284	540	19 900	21 900	15 600	12 000	18 800	16 600
	1 110	1 070	15 100	15 700	12 100	8 500	25 700	27 200
mv ±S.E.M	±206.0	±194.9	±3 720	±3 40	±3 020	±1 880	±7 000	±6 000

III. Formation of CO_2, Δ^1-pyrroline, GABA and the unknown compound(s) from [14]C-putrescine in fetal human placentas. Aliquots of placental tissue homogenate were incubated for 30 and 90 min. The sum of the products is also given. The figures are dpm/sample.

	Δ^1-pyrroline		GABA		Unknown compound( )		Sum	
...n 90 min	30 min	90 min	30 min	90 min	30 min	90 min	30 min	90 min
52	2 600	6 800	510	2 140	0	1 590	3 200	10 500
167	510	1 500	400	2 380	530	1 750	1 440	5 600
52	1 640	6 900	300	1 010	310	1 290	2 250	9 200
35	2 170	5 400	2 580	3 500	100	1 570	4 900	10 500
—	12 700	—	5 700	—	1 330	—	19 800	—
—	1 220	—	700	—	640	—	2 560	—

...is known that γ-aminobutyraldehyde is non-enzymatically converted into Δ -pyrroline that at steady-state the two compounds exist in equilibrium (Jakoby and Fredericks ...9). The method elaborated by Okuyama and Kobayashi (1961) measures only one of the ...ducts formed from putrescine, i.e. Δ^1-pyrroline. In the present study the amount of ...roline was found unrelated to the amount of tissue and the time of incubation. In addition ...he guinea-pig liver the pyrroline represented only a small fraction of the products formed ...m putrescine via the diamine oxidase pathway. It thus appears that the method of Okuy...a and Kobayashi (1961) should not be used indiscriminately for determination of diamine ...dase activity in tissues. This rapid method has been widely used, mostly for determination ...diamine oxidase activity in plasma. It has also been used for assay of diamine oxidase ...ivity in tissues utilizing homogenate or supernatant of tissue extract. For determination ...diamine oxidase activity in plasma the method appears safe as plasma lacks enzymes ...ich degrade γ-aminobutyraldehyde.

In the guinea-pig liver the major radioactive metabolite of putrescine found, besides ...ABA, was an unidentified compound(s). This is in agreement with our results after ad...nistration of [14]C-putrescine to mice (Hemmingsson and Rosengren 1976). Seiler and Eichen...f (1975) have observed urinary excretory products of putrescine, which they suggest are ...ontaneous reaction products of the highly reactive γ-aminobutyraldehyde or of Δ^1-pyrro...e. The unknown compound(s) observed in our study was not extractable in toluene and ...s not found when [14]C-putrescine was incubated with purified hog kidney diamine oxidase. ...ese facts together with the observation that the production of the unknown compound(s) ...s approximately proportional to the tissue amount and the time of incubation suggest to ... that the unknown metabolite(s) is formed enzymatically. In preliminary expts. formation ... the unknown compound(s) on incubating homogenate of guinea-pig liver with [14]C-GABA ...s negligible in spite of a high formation of [14]CO indicating the unknown compound(s) ...t being a metabolic product of GABA.

The production of GABA in relation to Δ -pyrroline was lower in the human placenta ...an in guinea-pig liver. The reason for this is so far not known. One possibility is that the ...lation between diamine oxidase and γ-aminobutyraldehyde dehydrogenase may be different ... the two tissues. Alternatively the dehydrogenase might be a more labile enzyme than dia-

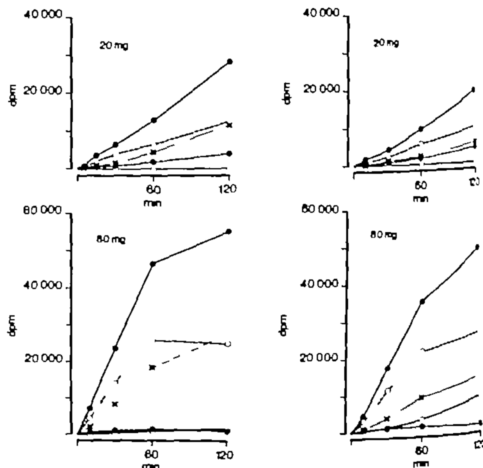


Fig. 2. Rate of formatio of different metabolites from [14]C-putrescine in guinea-pig r ... as related to time of incubation. The metabolites were CO_2 (O—O), Δ^1-pyrroline (●--●), GABA (O—O) and known compound(s) (× ×). The total amount of these metabolites is also given (●—●).

line formed in the samples was so high as to interfere with the determination of the ot products in the thin-layer electrophoresis system Results obtained on incubating hu fetal placenta are given in Table III

Discussion

The present in vitro study shows that mammalian tissues rich in diamine oxidase are cap of producing various compounds derived from putrescine compounds which recently h been found after in vivo administration of putrescine (Seiler *et al* 1971 Henningsson Rosengren 1976). Our present work also indicates that diamine oxidase is important in putrescine metabolism, one of the major metabolites formed being GABA. In vivo stu with [14]C-GABA have shown it to enter the tricarboxylic acid cycle (Roberts 1956, Wi *et al* 1959). Thus, a main route in the metabolism of putrescine seems to be a multi sequence with several enzymes involved where the first two steps of the route are catal by diamine oxidase and γ-aminobutyraldehyde dehydrogenase respectively In consid the production of CO as related to time of incubation, the multi-step sequence in the l way between putrescine and CO should be kept in mind

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Tension-length behaviour of a molluscan smooth muscle related to filament organisation

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Abstract

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…nder optimal activation conditions … obtain an active tension-length curve …ith … well-defined plateau …. The plateau can be explained in terms of … sliding filament mechanism in …hich double overlap of … thin filaments does not interfere …ith cross-bridge formation between thick and thin filaments of the …pposite polarity

…e shape of the tension-length curve in vertebrate skeletal muscle can be explained on the …ding filament theory according to which the tension developed is proportional to the …ount of overlap between the (thin) actin and (thick) myosin-containing filaments (Huxley …d Niedergerke 1954, Huxley and Hanson 1954). To provide a quantitative demonstration … this relation it is necessary to know the length of the thin and thick filaments, and to have … method of relating the tension-length curve to filament overlap (Gordon, Huxley and …lian 1966).

At first sight there would seem to be little chance of accounting for the tension-length …rve of a smooth muscle in terms of filament overlap. In the absence of a visible band …ttern there is no easy way of relating overlap to muscle length. Furthermore it is difficult … interpret the extent to which the behaviour of a whole muscle reflects the mechanical …roperties of its individual cells. Experiments with single smooth muscle cells have only been …tempted very recently and still present formidable difficulties. Another serious obstacle … that most smooth muscles respond to stretching by spontaneous contractile activity …Prosser 1974). On the structural side estimates for the length of thin and thick filaments …re so far available only for one type of smooth muscle, namely the anterior byssus retractor …f the mollusc *Mytilus edulis* (ABRM) (Lowy and Hanson 1962, Sobieszek 1973).

We report here experiments with the ABRM where we have established optimal stimula…tion procedures and eliminated spontaneous responses. Under such conditions the tension-…ength curve shows a feature hitherto seen only in frog skeletal muscle single fibre prepara-

mine oxidase. If so the activity of the dehydrogenase might be reduced during ... should be noted that diamine oxidase activity in human pregnancy reaches a plateau ... the 7th month of pregnancy with high values up to term (Ahlmark 1944). It has been ... in rats that the synthesis of putrescine is high in the placenta during the last trimester ... gestation (Kim and Harris 1975 Guha and Jänne 1976) Much remains to be ... regarding the role of diamine oxidase activity in the placenta not to mention other ... containing this enzyme.

We are grateful to the staff of the Department of Obstetrics and Gynaecology Lund, for their ... supplying human placental tissue. This study was supported by grants from the Swedish Medical Res... Council (Grant No. B77-04X-02212 10C) and from the Medical Faculty University of Lund.

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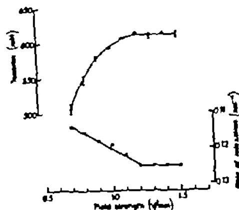
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1. Relation between stimulus strength and phasic contraction. The upper curve shows the dependence ... phasic tension on ... field strength for ... muscle maintained in ... 5 HT-containing sea ... water ... at 0.95 L_o. After mechanical and electrical equilibration the field strength ... varied between ... 0.7 and 1.5 V/mm and 5 constant responses were recorded at each particular setting. Vertical bars ±1 S.D. In the lower curve the relation of maximum relaxation rate to field strength for the same ... shown. The points here represent the mean of 2 measurements ... whose deviation ... less than 2% ... that relaxation rate and active phasic tension becomes constant at about the same field strength ... about 1.2 V/mm).

Results

Determination of optimal stimulus strength for isometric phasic contractions

...wy and Millman (1963) showed that stretching an unstimulated ABRM produces 2 kinds ...ension, apparent resting tension (ART) which is abolished by application of 5 HT and ... resting tension (RT) which is considered to be the tension present in a 5 HT-treated ...scle.

To produce phasic contractions, alternating current can be applied either to the untreated ...scle or to a muscle maintained in a 5 HT-containing medium. The most important differ... between these two preparations are that in the latter no ART is present and relaxation ...ppreciably faster (Lowy and Millman 1963).

...o produce a smooth 'tetanic' response the muscle was stimulated at 10 C through trans... electrodes at L_o with alternating current and a field strength of 1.5 V/mm. The stimula... period was 7 to 9 s. Intervals of 240 s were allowed between each period of stimulation. ... manner of stimulation was continued and eventually completely uniform responses ... obtained. Stimulation lasting longer than 7 to 9 s produced higher peak tensions (see ...tion 3), but the muscle fatigued, we therefore did not stimulate for longer than 7 to 9 s.

...nce uniform responses were obtained the field strength was varied at every muscle ...gth investigated and about 5 constant responses were recorded at each setting. Under ...se conditions a tension plateau was produced (Fig. 1 upper curve). The maximum tension ...veloped per cross sectional area at L_o (P_o in mN/mm²) depended on the size of the muscle, ...ng higher the smaller the muscle. Conceivably smaller muscles have relatively less colla...nous material. The maximum peak tensions measured varied from 600 to 1 200 mN/mm². ...n the same responses the maximum relative rate of isometric relaxation (s^{-1}) was deter

tions under special experimental conditions (Gordon *et al* 1966), namely a plateau; along which maximum tension is developed. We discuss various interpretations of ABRM plateau.

Methods

Very small specimens of *Mytilus edulis* were collected from local beaches and kept in an aquarium where aerated sea water was circulated at 4°C. The ABRM muscles used were about 10 to 15 mm long and weighed between 3 and 10 mg.

Dissection

The animal was opened by cutting the adductor muscles. The foot and soft organs were removed, leaving intact the two ABRM muscles and all the posterior byssus retractor muscles (PBRM). A very light gold chain was tied to the latter just behind the byssus stem. One of the ABRM muscles was cut near its insertion into the byssal mass, and all the posterior retractor muscles were cut a few millimetres distal to the gold chain. The shell to which the anterior end of the cut ABRM remained attached was removed; the other shell was chipped with bone forceps so as to leave only a small piece around the attachment of the intact ABRM

Mounting

The piece of shell was clamped into an adjustable perspex holder situated at the base of a perspex chamber in which the muscle was suspended vertically by connecting the gold chain through a micrometer arrangement to a strain gauge transducer The overall compliance of this set-up was 1.0 µm/mN In some experiments we used a myograph with a stepping motor (Mulvany 1975) to change the muscle's length automatically. The overall compliance of this set-up was 0.5 µm/mN Two types of chambers were used depending on whether the muscle was stimulated by electrical shocks or by Acetylcholine (ACh). The ACh chamber had a smaller volume so that it could be filled and emptied within 2 to 3 s.

Oxygenated sea water at 10°C was circulated through the chamber at a rate of about 5 ml/min. With a thermistor placed near the muscle the temperature was recorded throughout the experiment. Before the start of each experiment the muscle was mechanically equilibrated by allowing it to hang with a load of for 1 h. The osmolarity of the sea water was about 750 mOsm and its pH about 8. These did not change significantly in the course of an experiment.

Stimulation

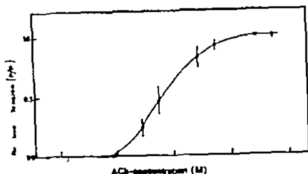
Depending on the method of stimulation the ABRM can produce two kinds of isometric responses. Repetitive electrical shocks cause a phasic contraction (Fletcher 1937) which is characterised by a fairly high relaxation rate. ACh induces a tonic 'catch' contraction (Twarog 1954) whose relaxation rate can be 2 orders of magnitude lower than in a phasic response (Lowy and Millman 1963)

To stimulate the muscle electrically alternating current was applied through transverse silver-silver chloride electrodes. The field strengths varied from 0.5 to V/mm, depending on the size of the muscle. We also used condenser shocks with a multi-electrode assembly (Jewell 1959), and square wave pulses end to end electrodes (Lowy and Millman 1963)

Twarog (1954) described how the ABRM can be stimulated with ACh and relaxed with 5-hydroxytryptamine (5-HT). For experiments where we have used these drugs they were both freshly dissolved in sea water

Muscle length changes

The muscle's length was measured in the chamber using a telescope with a calibrated eyepiece. We have taken as reference length (L_0) that length at which a small resting tension (approximately 1 mN) can be detected in the presence of 5-HT at a concentration of 2.5 × 10^{-4} M This concentration is the lowest that completely abolished apparent resting tension (Lowy and Millman 1963) throughout the expt. We found that if muscles were left in a sea water medium containing a higher 5-HT concentration they tended to deteriorate within a few hours. L_0 was established before the start of each experiment



[Fig.] 3. Relation between tonic tension and ACh concentration. The dose-response curve was obtained [from] experiments with 5 different muscles stimulated with ACh at L_o. The ACh concentration is given in [a] logarithmic scale. The tension is expressed relative to maximum active tension, the mean of [which] is 970 mN/mm². The vertical bars show ± 1 S D

[rapi]dly and always to the original level. Using a concentration of 5.5 10^{-4} M ACh the [mus]cle was stimulated at 20 min intervals until about 5 successive uniform responses were [obta]ined. Muscles were rejected unless they could be equilibrated in this manner

[U]sing such equilibrated muscles we determined the dose-response curve for ACh by apply[ing] various concentrations of ACh and found that a tension plateau was produced at ACh [concentrati]ons higher than about 3 10^{-4} M (Fig. 3). However we also found that con[cen]trations of ACh above 5.5 10^{-4} M caused irreversible damage. Therefore in our sub[seq]uent expts. on the muscle's active tension-length behaviour we only used ACh in a con[cen]tration of 5.5 10^{-4} M, which produced more than 90% of the maximum tension.

[The] Effect of length changes on the active tension production

[Ha]ving established optimal activation conditions for both phasic and tonic responses we [stu]died the muscle tension-length behaviour in both types of contractions. It is known from [pre]vious work that greater tensions are generated with ACh than with repetitive electrical [sho]cks (Jewell 1959). This suggests that ACh produces more complete activation and we [the]refore describe here mainly our expts. on the ABRM's length-tension behaviour for tonic [co]ntractions.

Using ACh stimulation we investigated if the active tension-length behaviour of the [AB]RM was reversible over the muscle's normal working range. The latter we found to be [be]tween 0.8 and 1.2 L_o from expts. where the ABRM and PBRM were stimulated *in situ* [(Fi]g. 4).

Fig. 5 illustrates the existence of active tension loss and plasticity two phenomena that [ha]ve been demonstrated previously in the ABRM (Abbott and Lowy 1958), as well as in [m]ammalian smooth muscles (Dobrin 1973 Petersen and Paul 1974), and possibly also in [the] obliquely-striated muscle (Miller 1975). The ABRM was stimulated with ACh once at [ea]ch particular length. Both active and resting tension were determined. Starting at about [0.]9 L_o, the muscle was lengthened, shortened and lengthened again as indicated by curves 1 [an]d 3 in Fig. 5. All length changes were effected in steps of 0.05 L_o. It may be noted that as

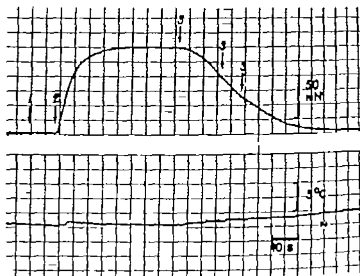


Fig. 2. Isometric tonic response of an equilibrated ABRM. Recorder trace of an ACh response at 10°C. The upper trace shows the active tonic tension developed at L_0 and the lower trace shows temperature variations. The calibrations are: tension 8.5 mN/div and temperature 0.5 C/div Arrows (1) emptying of chamber (2) application of ACh, (3) emptying of chamber and washing with 5-HT-containing sea water

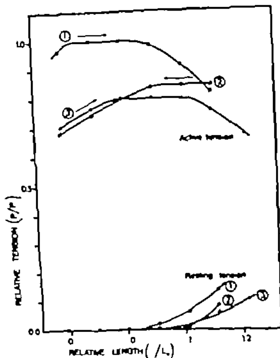
mined at each length as the slope of the relaxation phase of the tension-time curve at point of inflexion divided by the peak tension during the response concerned. We found the maximum isometric relaxation rate increased as field strength and tension was increased until a plateau was reached (Fig. 1 lower curve)

From these observations we conclude that for isometric phasic contractions at any particular muscle length the ABRM was stimulated optimally only when peak tension and relaxation rate reach plateau values. Using such conditions, completely uniform responses can be produced once every 240 s for periods up to 12 h. In previous work with the ABRM it proved impossible to obtain optimal activation (*i e* a plateau on the stimulus-response curve) for phasic responses using longitudinal field stimulation (Jewell 1959 Lowy & Millman 1963) With such stimulation we were also unable to obtain optimal stimulation. This is in line with results from experiments with frog skeletal muscle which have demonstrated that longitudinal field stimulation does not produce optimal stimulation (Sten-Knudsen 1960)

2. *Determination of optimal ACh concentration for isometric tonic contractions*

After the usual mechanical equilibration and determination of L_0 the muscle was maintained in a 5-HT-containing sea water medium (5-HT concentration 2.5 10^{-4} M) between applications of ACh. We found that in the absence of 5-HT it was difficult to obtain several successive uniform tonic responses after application of ACh it takes a long time before the original resting tension level is re-established, and muscles also tend to fatigue. We therefore adopted the procedure illustrated in Fig. 2.

Before each stimulation the 5-HT-sea water medium was replaced by a sea water ACh containing solution the resulting tonic response levels out at a plateau. As soon as the plateau was reached ACh was washed out and replaced by the 5-HT medium tension



Plasticity and tension loss in the ... stimulated with ACh. The muscle ... equilibrated at about 0.9 L_o using ... Tonic responses were obtained at ... lengths by stretching the ... passively in steps of 0.05 L_o up ... 1.15 L_o and stimulating with ACh ... 1, squares). The ABRM ... as then ... ed passively in steps of 0.05 L_o and ... active tensions developed by ACh ... lation ... re recorded (curve 2, ...). Finally the first procedure was ... ted and produced curve 3 (triangles) ... corresponding resting tension curves ... lso show ... For further details see ... In the active tension length curves ... the shift along the length axis ... icity) and the downwards shift ... the tension axis (tension loss).

...o test if activation is also involved in the phenomenon of tension loss we performed a ...s of expts. with ACh stimulation as shown in Fig. 6 a, b, c. The muscles were equili... ed at a certain length (reference length) and 3 to 5 constant responses were recorded ... in Fig. 6). After 10 min the muscle was passively stretched by 0.05 L_o, and after 10 min ... as again passively released to the reference length and 3 to 5 constant responses were ... ained (○ in Fig. 6). The same procedure was then repeated, but this time the muscle was ... ulated at the longer length (■ in Fig. 6) before being released to the reference length ... stimulated again (□ in Fig. 6).

...bove and near L_o it is evident that tension loss occurs when the muscle has been stim... ted at a longer length (● and □ in Fig. 6 b, c). This tension loss is probably not due to ... se slip as no tension loss occurs if the muscle is only passively stretched but not ... mulated ... t the longer length (and ○ in Fig. 6 b, c).

...elow L_o no such tension loss upon stimulation at the longer length takes place (Fig. 6 a). ...ether this tension loss above L_o upon stimulation at longer length is due to some kind o... ment rearrangement after which the system only "recovers" very slowly is currently unde... estigation.

These results demonstrate that for this particular kind of muscle it is impossible to obtai... sistent and reproducible tension-length curves over the natural working range for di... nt muscles unless a definite sequence in the length changes are performed.

Conditions in which a plateau in the active tension-length curve is obtained

...view of the above results we used one of the following two procedures to obtain the acti...

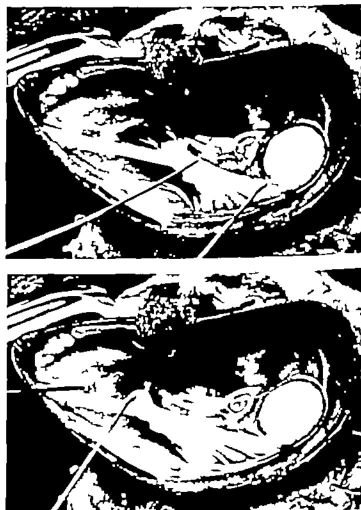
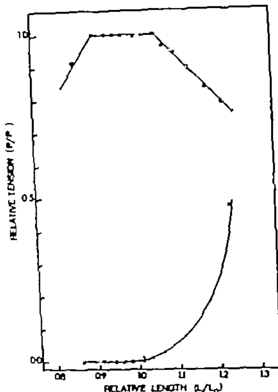


Fig. 4. The ABRM wo king range. The pictures show a *Mytilus* whose soft organs hav been re leaving intact the ABRM (to the left) and its a tagonists, the PBRMs (t the right). The foot and organ can also be seen. The upper picture shows a situation where the PBRM are being stimulated sively with a.c. through two silver electrodes After stimulation of the PBRM the ABRM was str (lower picture). The length of the shell is bout 30 mm.

resting tension was measured in the presence of 5-HT there is here no possibility of a tribution due to ART

The phenomena of active tension loss and plasticity are most convincingly demons by curves 1 and 2. Thus the active tensions produced at decreasing muscle lengths (ac tension curve 2) all are below those previously given at the same length during the first p of the expt. when the muscle s length was increased (active tension curve 1) It is also evi that in the second part of the expt. the position of the plateau has shifted to the right al the length axis. The opposite tendency is apparent when the muscle is lengthened agai active and resting tension curves 3) but this time the active tension loss is relatively sma

The observed lengthening of the muscle is very likely due to plastic slip between co genous elements This explanation is in line with the observation that the phenomenon is reversible.



Active and resting tension-length for tonic contractions. Results 3 muscles represented by 3 different ls (O ●). The muscles were ated at 10°C by ACh at concen- of 5.5 10^{-4} M. Length changes ps of about 0.05 L_o were effected micrometer device whilst the muscle completely relaxed. One ACh re- was obtained at each length. The g tension was measured in the ce of 5-HT as described in the text. oints indicated by the symbols ( ) (●) were obtained from muscles d from pre-stretched state whereas ts represented by the symbol (O) om muscle started from pre- ened state. The overall compliance set-up (including connections to muscle) was 3 μm/mN. The plateau 0.15 L_o.

n ascending limb, were active tension production increases up to an abrupt change at art of the plateau; (iii) a descending limb, whose onset is also abrupt.

ble I shows that the plateau characteristics (i) remain essentially the same in phasic tonic contractions and (ii) do not depend on whether the muscles used were in a pre ched or pre-shortened state. As regards (i), there is evidence indicating that the mech- m of calcium release is different in the two types of contraction (Sugi and Yamaguchi 6). Moreover we have already mentioned the possibility that the muscle may be more activated by ACh than by repetitive electrical shocks. Nevertheless, Table I shows the ence of a plateau under both kinds of activation procedures.

nder experimental conditions rather similar to ours the muscle's series elasticity has been mated to be between 0.03 to 0.05 L_o/P (Lowy and Millman 1963 Jewell 1959). We erefore assume that in our expts. the isometrically stimulated muscle could shorten by at t 0.05 L_o. T test the possible effects of such internal shortening on the existence of the ean we performed an expt. where the muscle was allowed an extra shortening of 0.023 L_o ing stimulation (quick release). Fig. 9 shows that this procedure left the plateau essenti- unchanged. The downward shift of curve 3 relative to curve 2 suggests that deactivation rs. the observed recovered tension loss resembles that seen in similar expts. with frog etal muscle (Edman 1975). Finally we found that the plateau was still present under ditions when tension loss and plastic slip occurred (Fig. 5).

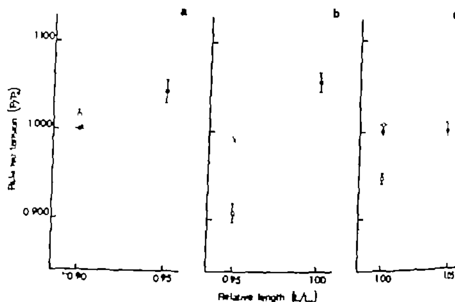


Fig. 6. Reversibility experiments. Experiments to illustrate how the tonic tension produced at a reference length is affected when the muscle has been passively stretched and then at the longer either stimulated or not stimulated. The reference tension (●) is compared with the tension produced after a passive stretch and subsequent release of 0.05 L_o (○), and the tension produced after stimulation at the longer length (□). Results from experiments performed at 3 different reference lengths are shown. Results from 5 muscles equilibrated at a reference length of 0.90 L_o. (b) Results from 6 muscles equilibrated at a reference length of 0.95 L_o. (c) Results from 3 muscles equilibrated at a reference length of 1.0 The points are the mea ± 1 S.E.

tension-length curve over the ABRM s natural working range (i) the muscle was started in pre-shortened state and its length was passively increased (ii) the muscle was started in pre-stretched state and its length was passively decreased.

Fig. 7 shows the active tension-length relation obtained for 3 muscles using ACh stimulation The muscle s length was passively changed 10 min after ACh was washed out, and after another 10 min. ACh was applied again at the new length Starting either below or above the muscles were lengthened or shortened in steps of 0.02–0 05 L_o.

The descending limb of the tension-length curve above the plateau was studied in detail in a series of expts with muscles started from an extremely pre-stretched state (Fig. As the muscles were all started from a pre-stretched state the points on the descending limb of the curve are unlikely to be affected by damage to the muscle which could conceivably be the case in expts. with muscles started from a pre-shortened state. Linear regression analysis of the points on the descending limb of the tension-length curve (from 1 05 to 1 4 L_o) gave a straight line with slope −1 11 ±0.04 (mean ± S.D) and a correlation coefficient 0.986. The intercept on the length axis of this regression line is 1 93 L_o ±0.03 L_o (i.e. the length at which no active tension would be developed).

We have determined the active tension-length curve for untreated and 5-HT-treated muscles and obtained the same results in both cases. The curves have the same general shape as that for tonic contractions (see Table I).

Whichever of the procedures was adopted all the active tension-length curves we obtained

1 Characteristics of the plateau region in active tension-length curves (phasic and tonic).

of action	Starting condition (1)	Number of experiments	Plateau range Lower limit (2)	Upper limit
	pre-shortened	6	0.90 ± 0.08	1.06 ± 0.06
	pre-stretched	6	0.85 ± 0.03	1.06 ± 0.03
	pre-shortened	4	0.9 ± 0.01	1.04 ± 0.01
	pre-stretched	7	0.93 ± 0.04	1.06 ± 0.04

ures are given as fractions of L_0 and are the mean ± 1 S.D. (1) Experiments started either at about (pre-shortened) or 1.2 L_0 (pre-stretched). (2) Plateau range is the range of muscle lengths over which to 1.00 P is developed. (P is the maximum tension at L_0 in mN/mm².) The muscle lengths at which the plateau begins (upper limit) and ends (lower limit) are indicated.

s that the whole muscle behaves functionally as a single cell, that the length of the thick thin filaments is constant, and that at any particular muscle length the 'sarcomeres' in cell have the same amount of filament overlap. The idea that under suitable activation ditions the ABRM could respond as a single cell is supported by recent work which s that, as in vertebrate smooth muscle, the cells in the ABRM are electrically connected exal junctions (Twarog, Dewey and Hidaka 1973). Electron microscopy (Sobieszek 3) indicates that the filaments in the ABRM are organised in an arrangement comparable sarcomere, and the available evidence does not exclude the idea that both the thick ments and the thin filaments have uniform lengths (Sobieszek 1973). In fact, in verte te smooth muscle it has been established that the length of the thick filament is constant Iliott, Somlyo and Somlyo 1975). The presence of sharp corners on the ABRM tension-gth curve makes it unlikely that the plateau can be explained as the sum of individual sion-length curves from cells whose lengths are statistically distributed.

We here show that the ABRM plateau can be explained on a sliding filament mechanism ically similar to that for striated muscle. That such a mechanism operates in the ABRM is ongly suggested by structural evidence both from electron microscopy (Lowy and Hanson 62) and X-ray diffraction studies (Lowy and Vibert 1972). Electron microscopy has also wn (Szent-Gyorgyi et al. 1971) that there is reversal of polarity in the middle of the thick ment, and that the thin filaments have opposite polarity on either side of a structure led dense body (which is equivalent to a Z line (Lowy and Hanson 1962)).

By analogy with the sliding model for frog skeletal muscle the simplest explanation for the BRM tension plateau would be in terms of a cross-bridge free region of sufficient length the middle of the thick filament (Gordon et al. 1966). Estimates for the lengths of the ick filaments in the ABRM vary between 10 and 30 µm (Sobieszek 1973 Lowy and Hanson 62), and the ABRM plateau would therefore require region free of cross-bridges of 3–4.5 µm. Such regions have not yet been found and we consider this possibility to be likely on structural grounds.

In mechanically skinned cardiac fibres Robinson and Winegard (1977) found that the ngths of thin filaments vary by as much as 0.6 µm within single sarcomere in a single

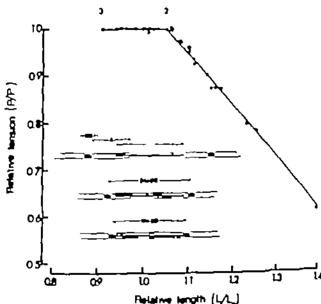


Fig. 8 Relation between active tensio and muscle length for tonic responses. The points ... from experiments with 6 different muscles stimulated by ACh at a concentration of 5.5 10^{-4} M ... in steps of 0.05 L_0 was effected by a micrometer device One ACh response was obtained at each ... The overall compliance of the set-up (including connections to the muscle) was 1 μm/mN ... obtained from experiments started with muscles in a pre-stretched state are shown. Also shown ... figure is the length-tension curve (full line) predicted from the model illustrated in Inset. The ... straight line of the descending limb of the predicted length-tension curve is -1.20 ± 0.06 S.D (The ... here is due to the experimentally found variations in plateau range, see Table I.) Linear regression ... of the poi ts obtained over that region of muscle lengths (from $L/L_0 = 1.05$ to 1 40) gives a ... with slope $-1\ 11 \pm 0\ 04$ S.D (The length t which no active tension would be developed as calculated ... the experimental points is 1.93 $L_0 \pm 0.03$ L_0.) The numbers and arrows along the top refer to the ... in the Inset

Inset Sliding f lament model to explain the ABRM plateau. The drawings show schematically the ... arrangement in sarcomere at critical stages in the muscle s length-tension behaviour (1) Diagram ... sarcomere. *A* length of thin f lament on one side of dense body *M* length of thick filament, ... length of the dense body (2) Arrangement of filaments i a sarcomere at the beginning of the ... (equivalent to 1 05 L_0 in Fig. 8 nd upper limit in Table I). Here the thin filaments just meet ... of the sarcomere The length of the sarcomere is 2A + DB. (3) Arrangement of f laments at the end ... plateau (equivalent t 0 91 L_0 in Fig. 8 nd lower limit in Table I). The ends of the thick filaments ... the dense bodies. Sarcomere length is M + DB.

Discussion

The finding of a plateau with sharp corners on the active tension length curve of a ... smooth muscle is remarkable and suggests a highly ordered filament arrangement ... ABRM muscle cells. The relative length at which this plateau begins and ends is ... same for tonic and phasic contractions and for muscles started in a pre-shortened ... stretched state (Table I). A plateau is also present when the muscle is deactivated by ... release (Fig. 9) and under conditions where tension loss and plasticity occur (Fig ... presence of a plateau under these various conditions leads us to attempt an explanation ... it in terms of events at the filament level To do this we require a mechanism by which ... the plateau range, changes in muscle length are not accompanied by changes in the ... of cross-bridges formed between the thin and thick filaments.

... on either side of the plateau of the ABRM tension-length curve ...

—minates when the dense bodies reach the ends of the thick filaments (but not necessarily them). Here the ends of the thin filaments begin to overlap thick filaments of the 'wrong —rity right from the beginning of the plateau.

—s in the frog model we assume that cross-bridges cannot form with thin filaments that —lap thick filaments of the 'wrong' polarity. In the frog model the abrupt loss of tension —ch occurs right after the end of the plateau is believed to be due to interference with cross- ge formation when 'double overlap' begins because of strong mechanical constraints —e highly regular filament lattice of the frog muscle (Gordon *et al.* 1966, Huxley 1965). —he ABRM there are fewer such constraints, there is no regular lateral packing of the —k filaments (Sobieszek 1973, Lowy and Hanson 1962) and for each thick filament there —about 17 thin ones (Sobieszek 1973) and some of these do not surround thick filaments, —he in small lattices between them, as in —ertebrate smooth muscle (Ashton *et al.* 1975). —believe that in both these types of smooth muscle spatial considerations will not lead to —rference with cross-bridge formation at 'double overlap'.

—sing the data from ACh contractions, then according to our explanation the upper —t of the plateau (see Table I) is equivalent to a sarcomere length of 2A — DB (where A is —length of the thin filaments on one side of the dense body whose length is denoted DB) — 2A + DB = 1.05 L_o. The lower limit of the plateau (see Table I) is equivalent to a sarco- —re length of M — DB (where M is the length of the thick filament): (2) M + DB = 0.91 L_o. —m electron microscope data (Sobieszek 1973) we take: (3) 2A/DB = 12.3. From (1), (2), —e then obtain the non-overlap length, 2A + M — DB = 1.88 L_o and the ratio of thin to —ck filament length, A/M = 0.58.

—ssuming that the cross-bridges are evenly distributed along the thick filament, the —cending part of the active tension-length curve for the frog model will be a straight line —ordon *et al.* 1966). Making the same assumption for the ABRM, the slope of this line —n be derived from the calculations above which give 1.88 L_o as the length of the stretched —scle where there would be no overlap. Fig. 8 shows that the experimentally determined —ints lie reasonably close to the calculated curve. Unfortunately the figure of 1.88 L_o —not be checked by expts. with whole muscle because the resting tension rises very steeply —ove 1.2 L_o (Fig. 2) and the muscle breaks when stretched to lengths greater than about — L_o.

We are aware that there are other plausible explanations of the ABRM's tension-length —haviour including the plateau. Thus in the absence of constraints such as an M-line or a —to-skeleton, the filaments might undergo some kind of rearrangement relative to one —other as the muscle is stretched. 'Shearing' (Rosenbluth 1972) or a mechanism that enables —ick filaments to change their thin filament partners (Poulsen and Lowy 1975) could account —r the ABRM plateau. But such mechanisms require much more elaborate assumptions —an the proposed model.

—e are greatly indebted for many stimulating discussions and suggestions to our colleagues Mike Mulvany —an Poulsen and John Wray. We also thank Lynne Monkton, Reginald Bernardino and Michael Stoltus — excellent technical assistance. This —ork was supported by — grant from the Muscular Dystrophy Asso- —ation of America.

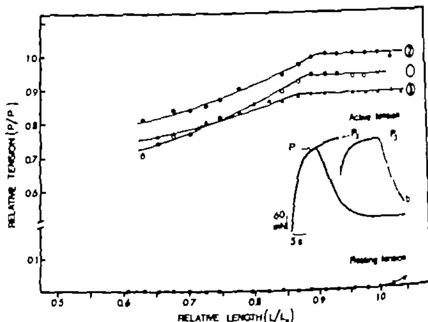


Fig. 9 Active and resting tension-length curves obtained from a quick-release experiment. The m pre-stretched to 1.05 L_o and stimulated by alternating current for 9 s every 240 s until it produced responses. It was then stimulated for 35 s and released after 20 s by 0.023 L_o at speed of 50 mm This procedure was repeated until the muscle had shortened to about 0.6 L_o. The myograph and same as that described by Mulvany (1975). The compliance of this set-up (including connecti muscle) was 0.5 μm/mN All tensions are taken as relative to the maximum peak tension obtained response at L_o (P in insert). Curve 1 represents the mean of 2 uniform contractions in a 9 s re in insert). Curves 2 and 3 were obtained using, respectively the 20 s and 35 s response (P and P

Insert Oscilloscope record from a quick-release experiment. Trace a Normal phasic respons stimulus. Trace b Response t a 35 s stimulus after 20 s the muscle was released by 0 023 L_o Normal phasic response at the shorter length which is identical to Trace 1

myofibril and this led them to conclude that the active tension produced in their prep depends not only on overlap but also on the distribution of lengths of the thin fi Active tension-length curves derived on the basis of these considerations show a about three times as wide as that obtained in isolated whole skeletal muscle fibres. H the corners between the plateau and the ascending and descending limbs of their t tension-length curves are very rounded The presence of thin filaments having length is therefore not very likely to account for the ABRM plateau we have obse

The ABRM plateau can, however be simply explained by a sliding filament mo resembling that in frog skeletal muscle except that double overlap of the thin fila the ABRM does not interfere with cross-bridge formation between thick and thin f of the appropriate polarity In what follows this explanation will be discussed with to the sliding model for frog skeletal muscle (frog model) (Huxley and Niederger Huxley and Hanson 1954 Gordon *et al* 1966). In the frog model the plateau begins respectively when the thin filaments reach the beginning and the end of the cross-br region. Further shortening brings the ends of the thin filaments to overlap thick fila the 'wrong polarity ('double overlap') According to our explanation (Fig. 8 In ABRM plateau begins when the thin filaments reach the middle of the thick filam

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Prostaglandin content in blood and lung tissue during alveolar hypoxia

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Abstract

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...im of the present work was to investigate whether prostaglandins (PGs) are synthetized and released ...n isolated blood-perfused rat and cat lungs secondary to vasoconstriction induced by alveolar hypoxia. ... lungs were perfused with autologous blood with constant volume inflow via the pulmonary artery in ...circulating system. They were ventilated with constant volume positive pressure, and acute alveolar ...xia was induced by ventilation with gas containing 2% O_2. A superfusion bioassay technique was ...d to measure PG-like activity in the perfusate from the lungs, the blood being re-oxygenated before ...ching the assay tissues. The oxygenator prevented the perfusate hypoxia induced by ventilation hypoxia ...affect the bioassay tissues. The assay tissues were rat stomach strip, rat colon and chick rectum. They ...re sensitive to calibrating doses of 0.5–1 ng/ml PGE_2 and 1–2 ng/ml $PGF_{2\alpha}$. In another series of experi...ents PGs of the F-series were measured in lung tissue from normoxic and hypoxic lungs with radio-...immunoassay techniques. No increase in PG-like activity could be detected in the venous effluent by means ...bioassay during hypoxia, nor was the lung tissue content of immunoreactive PGF increased by hypoxia. ...present findings indicate that alveolar hypoxia does not stimulate PG-synthesis in lungs, refuting that ...s are important mediators of the pulmonary vasoconstrictor response to alveolar hypoxia. It is concluded ...at PGs play no significant role in producing the pressor response to alveolar hypoxia.

...he mechanism whereby acute alveolar hypoxia elicits pulmonary vasoconstriction still ...mains to be properly clarified. Conflicting observations have been reported concerning a ...le for prostaglandins (PGs). These substances are possible mediator candidates for the ...ressor response to hypoxia since the lungs have a large capacity for synthesis and release ...f PGs secondary to various mechanical and chemical stimuli (Piper and Vane 1971, Piper ...1974). Moreover $PGF_{2\alpha}$ as well as various PG-endoperoxide analogs are potent constrictors ...n the pulmonary circulation (Änggård and Bergström 1963, Ross *et al.* 1976). Furthermore, ...ntrapulmonary synthesis of PGs induced by infusion of arachidonic acid also causes vaso-...constriction in the pulmonary circulation (Wicks *et al.* 1976).

Said and coworkers (1974) reported release of PG-like substances from isolated, perfused ...at lungs during ventilation with a hypoxic gas mixture. They also found that aspirin, which ...inhibits PG-synthesis (Vane 1973), reduced the vasoconstriction induced by hypoxia in

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TENSION TRANSDUCER
1
2
3
OXYGENATOR
Weight transducer
Pump 10 ml/min
37°C
5% CO_2+O_2
Pump 10ml/min
37°C
INJECTION POINT
RSS
RC
CR
Trachea
Pulm. vein
Pra
Pulm. artery
Lungs
Reservoir
37°C
Pump

1 Diagram of the experimental arrangement. The lungs are freely suspended in string fastened to heart inside heated (37°C) perspex chamber (not shown). They were ventilated with 5 CO_2 in air in control situation and 2 O_2, 5 CO_2 in N_2 during hypoxic tests. The lungs were perfused in re-circulating system via the pulmonary artery (Pulm. artery). Inflow pressure was recorded in sidearm of inflow cannula (P_{pa}). In the perfusion circuit the perfusate was heated (37°C) in the reservoir and before entering the lungs by the use of water jackets. With rat lungs flow of 10 ml/min was used, with lungs from 152 to 240 ml/min, constant for each preparation. With rat lungs the whole perfusate flow ml/min) was pumped to the oxygenator. With cat lungs 10 ml/min of the venous effluent was pumped to the oxygenator, the rest of the effluent perfusate was drained directly to the reservoir. The oxygenator heated (37°C) with water jacket. Blood was delivered to the top of the oxygenator and then drained down the internal rotating tube in contact with the oxygenator gas (5 CO_2 in O_2) flowing in the counter direction. The oxygenated blood then arrived in small semi-closed reservoir and from this it pumped by roller pump (10 ml/min) to the bioassay system. On the tubing was an injection point for testing infusions of PGs. The blood was again heated (37°C) before reaching the assay tissues: rat stomach strip (RSS), rat colon (RC) and chick rectum (CR). The tissues were superfused in series, the blood then drained to the main reservoir in the perfusion circuit. The isometric tension of the assay tissues continuously recorded. For further details, see Methods.

were inflow (152–240 ml/min) in recirculating system, with variable speed peristaltic pump, model &, Harvard Apparatus Co., Dover, Mass., USA. The weight of the preparation was continuously recorded. The lungs were suspended by the string around the heart, and connected to Sanborn force transducer model FTA-100-1. The other side of the transducer was connected to counter balance equipment. The transducer was connected to 7402A Hewlett Packard Recorder. The perfusion circuit schematically shown in Fig. 1.

Two cat lungs not included in Table I were used for testing the effects of the bioassay system including oxygenator on the pressor response to ventilation hypoxia (Fig. 2).

Since left atrial pressure and perfusate flow were kept constant, both in cat and rat lungs, changes in pulmonary vascular resistance were recorded as changes in mean inflow pressure (ΔP_{pa}).

Ventilation. After one or two gentle inflations of the lungs positive pressure ventilation was started, using Starling "Ideal" ventilation pump (C. F. Palmer, Ltd.). End-expiratory pressure was kept at about 2 cm H_2O by means of water seal. Stroke volume was adjusted to give peak tracheal pressure of about 6 cm H_2O at the onset both in rat and cat lungs. A ventilation frequency of 60 per min was used for rat lungs and 20 per min for cat lungs. The standard gas mixture used for ventilation was 5 CO_2 in air. Alveolar hypoxia was induced by ventilating the lungs with gas mixture of 2% O_2, 5 CO_2 and 93 N_2 for periods 3–10 min. The pH of the perfusate was measured with Radiometer acid-base analyzer (Model PHM 71). Perfusate pH was kept between 7.40–7.45 by supplying 5% CO_2 in air over the perfusate surface in the reservoir.

Oxygen tension in perfusate samples was measured with an oxygen electrode (PO_2-module, PHA 930, Radiometer) connected to the Radiometer acid-base analyzer.

anesthetized cats, and they suggested that vasoconstrictor PGs mediate this response ... and Hauge (1975) argued against this hypothesis and reported experiments on rat ... (Vaage, Bjertnæs and Hauge 1975) which showed that 3 different inhibitors of PG ... had no effect on the response to acute hypoxia in lungs from this animal. In a reply Sa... Hara (1975) reported additional experiments which again gave as a result that ... not indomethacin) reduced the pressor response to acute alveolar hypoxia. They sugg... that the conflicting results reported by Vaage, Bjertnæs and Hauge (1975) could be ... butable to species differences.

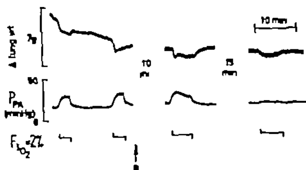
Anderson *et al* (1972) and Even *et al* (1975) reported that no increase in PG conten... found in blood neither from calves nor from humans during hypoxia-induced pulm... hypertension Furthermore, we and others have previously demonstrated that when ... synthesis is inhibited in different types of lung preparations from several species the ... monary vasoconstriction evoked by alveolar hypoxia was somewhat increased (Va... Bjertnæs and Hauge 1975 Tyler *et al* 1975 Weir *et al* 1976 a, Hales *et al.* 1977 Even *et al* 1975) It has therefore been suggested that the response to hypoxia is modulated by ... pulmonary synthesis and release of vasodilatory PGs (Vaage *et al* 1975). Thus, ... the evidence so far suggest that PGs does not mediate the vasoconstrictor response ... rather act to reduce it, preventing the pulmonary arterial pressure to become excess... high

In the present work we have aimed at determining once and for all the possible role ... for vasoconstrictor and vasodilator PGs in the pressor response to alveolar hypoxia ... have included cat lungs in addition to rat lungs and we have utilized a bioassay tech... similar to that of Said *et al* (1974) In addition we have determined lung tissue conte... PGF with radioimmunoassay Preliminary reports of this study have previously been pre... sented (Bjertnæs *et al* 1976, Vaage *et al* 1976)

Methods

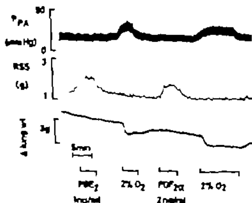
Rat lung preparation. 1 bread albino rats (local strain), weighing between 180–290 g, were used The ... donor animal was anesthetized with pentobarbitone sodium i p (3–4 mg/100 g b.w.t.), a tracheotomy ... performed, and the chest opened during positive pressure entilation. The trachea, the lungs ... larger intrathoracic vessels were dissected free and heparin was injected (100 I U in 0 5 ml saline) ... left ventricle before ligation of the caval veins. Stainless steel cannulas were placed in the pulmonary artery and the left atrium. The cannulas were fastened by a tight ligature around the heart, and the ... were transferred to a constant temperature (37°C) perspex chamber The lungs were freely suspended ... a string fastened to the heart ligature and perfused i ... recirculati g system with 40–50 ml hepar... (10 I.U./ml) blood, obtained by cardiac puncture of ether-anesthetized donor rats. All the four pairs of lungs which were used with bioassay were perfused w th a olume flow of 10 ml/min at the pulmonary artery by means of a roller pump. The 5 lungs used for radioimmunoassay had a flow of 15–20 ml ... Perfusion was begun within 12 min after the interruption of the nimals own circulation. Pulmonary art... pressure (P_{PA}) was measured by St tham P 23Db pressure transducer co nected to a Grass polygr... (Model 7B). A schematic drawing of the experimental set-up shown in F g. 1

Cat lung preparation. Cats weighing 1.9 t ... 1 kg were used as lung donors. They were nesthetized ... pentobarbitone sodium i.p (30–40 mg/kg b.w.t.). The lungs were dissected free as described for rat lu... As blood donors were used cats (b wt. 2.5–4.5 kg) f each perfusion expt. Also the blood donors w... anesthetized with pentobarbitone. They were heparinized w th 500 I U./kg i. ... od exsanguinated ... cardiac puncture. To each 100 ml of blood was then added 1 000 I U heparin dry powder (LEO). ... proximately 200 ml of blood was used in the perfusion circuit The cat l ... re perfused



The figure demonstrates the effect on the pressor response to hypoxia in rat lungs of including the oxygenator and bioassay circuit in the perfusion system. This particular preparation was studied for methodological reasons and is not included in Table I. From the top are illustrated change in preparation weight (Δ lung wt) and pulmonary arterial pressure (P_{PA}). The began with the preparation weight was ... (a regular observation). Two 3 min periods of alveolar hypoxia (F_{IO_2} = 2%) elicited good pressor responses when only the perfusion circuit was open. At point B the oxygenator-bioassay circuit was included. Ten min later a 5 min period of hypoxia still caused a pressor response, but the peak pressure was not maintained. After 36 min the pressor response to hypoxia had almost vanished. The weight reduction, related to changes in pulmonary blood volume, caused by alveolar hypoxia was better maintained and could be an independent type of response.

The pressor response to alveolar hypoxia in isolated rat lungs follows a characteristic pattern (Hauge 1968 a, b). At the beginning of perfusion standardized "hypoxic tests" produce no or very small pressor responses. After a few tests the response gradually increases until there is a maximum effect for the given time period of hypoxia. Several equal maximum responses can then be obtained before a gradual decline in responsiveness occurs,



1. Effects of ventilation hypoxia on isolated rat lungs perfused with blood at constant volume inflow. At the top are demonstrated recordings of pulmonary arterial pressure (P_{PA}), tension of the most sensitive bioassay tissue, rat stomach strip (RSS), which was superfused by the venous effluent, and changes in preparation weight (Δ lung wt). First the bioassay tissue is calibrated with 1 ng/ml of PGE_2 infused directly into the superfusing blood. Then comes a 4 min period with alveolar hypoxia (2% O_2), causing an increase in P_{PA}, this is the "classical" pressor response to acute hypoxia. Hypoxia also induced a rapid and reversible reduction in preparation weight, but had no effect on the RSS. After this period of hypoxia a stimulating dose of 2 ng/ml of $PGF_{2\alpha}$ was infused. Finally the effects of a 10 min period of ventilation hypoxia (2% O_2) is demonstrated. Again there is marked pressor response and a reversible reduction in preparation weight without detectable effect on the bioassay tissue.

Oxygenator. The blood superfusing the bioassay tissues (10 ml/min) was pumped through an oxy- to the bioassay tissues (Fig. 1). With cat lungs 10 ml/min of the venous effluent was pumped to the genator, the rest of the perfusate flow was shortcircuited to the inflow reservoir. Using rat lungs the perfusate outflow (10 ml/min) was passed through the oxygenator and was used for bioassay.

The oxygenator was made in The Mechanical Workshop, The Preclinical Institutes, Univer- Oslo. It consisted of a glas tube with a sinuous internal surface rotating inside a water jacket (37°C). internal surface of the tube was siliconized with Siliclad (Clay Adams, N. J., USA). The axis made an angle of 30 degrees with the horizontal plane. Thus blood delivered to the top of the tube was spread on the internal surface of the tube and then running slowly downward to a thermostated reservoir. of this semiclosed reservoir the oxygenator gas was delivered through a tube, the gas containing in O_2. The gas flowed upwards inside the internal lumen of the sinuous tube at a rate of 5 l/min. The wall of the tube was covered with a blood film. Gas and blood came in contact during counter- flow. The gas outlet was on top of the oxygenator, the blood outlet being at the bottom (Fig. 1).

PO_2 in the venous effluent decreased from 110–120 mmHg in the control situation to approx. 30 mmHg during ventilation with the hypoxic gas mixture. The oxygenator, however, maintained a constant PO_2 (500–525 mmHg) in the blood superfusing the assay tissues regardless of the shunt.

Bioassay. A modification of the superfusion organ technique (Vane 1969) was used for the assay of PGs. Our bioassay arrangement has been described in detail by Vaage, Scott and Wiberg 1978.

When leaving the oxygenator the blood was pumped by one channel of the roller pump (10 ml through a silastic tubing, with an injection point for infusion of standard doses of PGs. The superfusate was heated with a water jacket to 37°C before reaching the assay tissues (Fig. 1). The assay tissues were rat stomach strip (RSS), rat colon (RC), and chick rectum (CR) (Fig. 1). Before blood superfusion the tissues were treated for 2 h in a Krebs-Ringer solution containing pharmacological blockers against the action of catecholamines (α- and β-receptors), histamine (H_1-receptors), serotonin and acetyl- choline (Vaage *et al.* 1978). The isometric tension of the assay tissues was measured with sensing elements (Vaage *et al.* 1978). After passing over the tissues the blood was returned to the reservoir.

Calibrating doses of PGs were infused at the injection point. The assay tissues contracted to 0.5–1 of PGE_2 and 1–2 ng/ml of $PGF_{2\alpha}$.

Radioimmunoassay. 5 additional rat lungs were prepared as described and perfused with homol. blood but without the circuit for bioassay and the oxygenator. 4 min tests of alveolar hypoxia with of 5 min were performed throughout the perfusion period. When a marked pressor response was 3 pairs of rat lung were rapidly removed from the perfusion circuit after $3\frac{1}{2}$ min of alveolar hypoxia frozen in liquid freon cooled to about −140°C. 4–5 s passed between the stop of perfusion and the of the lungs to liquid freon. Two other rat lungs were perfused for similar periods of time and under normoxic conditions. They served as controls and were processed exactly as the hypoxic. Approximately half of the weight of the frozen preparation was used for tissue processing. Only the pheral part of lung tissue was used, thus avoiding large blood vessels and large airways.

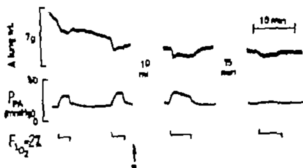
The lung tissue was homogenized with a Dounce homogenizer (2 min, 0°C) in 5 ml 0.15 M NaCl N HCl (5:2) giving a weight concentration of tissue of 200 mg/ml. Extraction of PGs was performed fraction of the homogenate (1 ml) that was transferred to tubes containing 2.6 ml ethyl acetate (1:1) and mixed on a whirlmixer (10 min). To each tube was added 3 ml 0.15 M NaCl and 2 ml ethyl and the tubes were centrifuged (50 000 g, 30 min, 4 C). A portion (3 ml) of the ethyl acetate phase evaporated under nitrogen at 50°C, the residue dissolved in 2.5 ml 0.01 M Tris-HCl (pH 7.4) and aliquots 50–150 µl taken for radioimmunoassay.

Before the labelled $PGF_{2\alpha}$ (New England Nuclear Corporation) was used for assay incubation, it purified on silicic acid columns (0.5 g, 100 mesh). The antiserum did not distinguish between the F com- pounds but showed no cross reaction with PGA, PGB, PGE_1, PGE_2, nor with the dihydro- et keto- tabolites of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ (Gautvik *et al.* 1 preparation).

All determinations were carried out in triplicates which differed less than 15%.

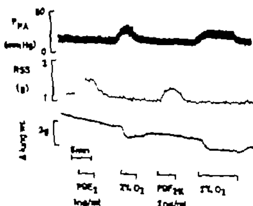
Results

Baseline values of pulmonary arterial pressure (P_{PA}) and flow as well as pressor responses to alveolar hypoxia are given in Table I. In cat lung preparations acute weight reduction ranging from 0.4 to 1.3 g occurred in all the periods with hypoxia concomitant with increase in P_{PA} (Figs. 2 and 3).



The figure demonstrates the effect on the pressor response to hypoxia in cat lungs of including the [illegible] and bioassay circuit of the perfusion system. This particular preparation was studied for [illegible] reasons and is not included in Table I. From the top are illustrated change in preparation (Δ lung wt.) and pulmonary arterial pressure (P_{PA}). T[illegible] began [illegible] the preparation weight was [illegible] regular observation). Two 3 min periods of alveolar hypoxia (F_{IO_2} = 2%) elicited good pressor [illegible] when only the perfusion circuit was open. At point B the oxygenator-bioassay circuit was [illegible]. Ten min later [illegible] 5 min period of hypoxia still caused a pressor response, but the peak pressure [illegible] [illegible]. After 16 min the pressor response to hypoxia had almost vanished. The weight reduction, [illegible] as changes in pulmonary blood volume, caused by alveolar hypoxia was better maintained and [illegible] be an independent type of response.

[illegible] pressor response to alveolar hypoxia in isolated rat lungs follows a characteristic [illegible] (Hauge 1968 a, b). At the beginning of perfusion standardized "hypoxic tests" [illegible] no or very small pressor responses. After a few tests the response gradually in[illegible] until there is a maximum effect for the given time period of hypoxia. Several equal [illegible] responses can then be obtained before a gradual decline in responsiveness occurs,



[illegible] Effect of ventilation hypoxia on isolated rat lungs perfused with blood at constant volume inflow [illegible] the top are demonstrated recordings of pulmonary arterial pressure (P_{PA}), tension of the most [illegible] bioassay tissue, rat stomach strip (RSS), which was superfused by the venous effluent, and changes [illegible] preparation weight (Δ lung wt.). First the bioassay tissue is calibrated with 1 ng/ml of PGE_2 infused [illegible] into the superfusing blood. Then comes [illegible] 4 min period with alveolar hypoxia (2% O_2), causing an [illegible] in P_{PA}. This is the "classical" pressor response to acute hypoxia. Hypoxia also induced [illegible] rapid [illegible] reversible reduction in preparation weight, but had no effect on the RSS. After this period of hypoxia [illegible] calibrating dose of 2 ng/ml of $PGF_{2\alpha}$ was infused. Finally the effects of [illegible] 10 min period of ventilation [illegible] (2% O_2) is demonstrated. Again there is marked pressor response and a reversible reduction in [illegible] weight without detectable effect on the bioassay tissue.

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Radioimmunoassay. 5 additional rat lungs were prepared as described and perfused with homologous blood but without the circuit for bioassay and the oxygenator. 4 min tests of alveolar hypoxia with intervals of 5 min were performed throughout the perfusion period. When a marked pressor response was present 3 pairs of rat lung were rapidly removed from the perfusion circuit after 3½ min of alveolar hypoxia and frozen in liquid freon cooled to about −140°C. 4–5 s passed between the stop of perfusion and the exposure of the lungs to liquid freon. Two other rat lungs were perfused for similar periods of time and frozen under normoxic conditions. They served as controls and were processed exactly as the hypoxic lungs. Approximately half of the weight of the frozen preparation was used for tissue processing. Only the peripheral part of lung tissue was used, thus avoiding large blood vessels and large airways.

The lung tissue was homogenized with a Dounce homogenizer (2 min, 0°C) in 5 ml 0.15 M NaCl and 1 N HCl (5:2) giving a weight concentration of tissue of 200 mg/ml. Extraction of PGs was performed on a fraction of the homogenate (1 ml) that was transferred to tubes containing 2.6 ml ethyl acetate/isopropanol (1:1) and mixed on a whirlmixer (10 min). To each tube was added 3 ml 0.15 M NaCl and 2 ml ethyl acetate and the tubes were centrifuged (50 000 g, 30 min, 4°C). A portion (3 ml) of the ethyl acetate phase was evaporated under nitrogen at 50°C, the residue dissolved in 2.5 ml 0.01 M Tris-HCl (pH 7.4) and aliquots of 50–150 µl taken for radioimmunoassay.

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Results

Baseline values of pulmonary arterial pressure (P_{PA}) and flow as well as pressor responses to alveolar hypoxia are given in Table I. In cat lung preparations acute weight reduction ranging from 0.4 to 1.3 g occurred in all the periods with hypoxia concomitant with increase in P_{PA} (Figs. 2 and 3).

r hypoxia. During the recordings of pressor responses in cat lungs reversible reduc- lung weight occurred concomitant with the increase in pulmonary arterial pressure 2 and 3). To our knowledge this effect has not previously been published. Such weight ges most likely represent reversible reductions in blood volume (Hauge *et al.* 1967 a). tion of pulmonary blood volume has been observed in intact animals during hypoxic ation (Aarseth, Karlsen and Bø 1975 Karlsen and Aarseth 1976 and Aarseth and n 1977). From Fig. it appears that when the pressor response has almost disappeared, is still a significant weight reduction during hypoxia. This may indicate that a reduc blood volume due to vasoconstriction of capacitance vessels occurs independent of ressor response. Similar dissociation between resistance and capacitance vessels in as previously been described (Hauge *et al.* 1967 b).

2 also illustrates the finding that when the bioassay circuit is opened the pressure se caused by alveolar hypoxia rapidly declines, allowing only a few good pressor nses to be elicited with the bioassay system in action. This observation should be d in context with that of Duke and Killick (1952). They perfused cat lungs for as long h with continuously increasing pressor responses to hypoxia. We ha e no definite nation for this discrepancy. Changes in the blood as it comes into contact with the gn surfaces of the oxygenator and the assay tissues may play a role. Reeves and Grover) have reported that small amounts of endotoxin block acute pulmonary hypertension ed by hypoxia in dogs. The assay tissues may certainly contain enough endotoxin of ral origin to block the hypoxic response.

e effect of endotoxin has been suggested to be mediated through synthesis of vaso- r PGs (Weir *et al.* 1976 b). However we did not find any evidence of a progressive e of PGs during perfusion in our setup. Contact of the perfusate blood with foreign ces such as polyvinylchloride (PVC) will also prevent the response (Duke and Vane)

e now turn to our main findings, namely those regarding pulmonary PG-synthesis g hypoxia. Perfused rat and cat lungs have considerable capacity to release PGs ndary to a variety of stimuli (Bakhle and Vane 1974). Whether intrapulmonary synthesis release of PGs will cause a constrictor or a dilator response depends on the balance reen the different subgroups of prostaglandins which are released. The release of PGs e lungs is dependent on a rapidly induced biosynthesis, since only negligible amounts of are stored in lung tissue (Piper and Vane 1971).

ur results show that in both rat and cat lungs perfused with blood, the vasoconstriction ed by ventilation hypoxia is not accompanied by detectable PG-synthesis. From studies perfused cat lungs Said and coworkers (1974) on the other hand, claimed that bioassay ponses during alveolar hypoxia "suggested the appearance in the perfusate of PGE-like pounds, with the activity of up to 50 ng of PGE_1 per milliliter and the presence of F-like compounds, with the activity of up to 50 ng of $PGF_{2\alpha}$ per milliliter" during ventila- hypoxia. An important methodological objection is that these investigators did not use xygenator and thus exposed the assay tissues to the direct effects of hypoxia (Said *et al.* 4, Fig. 1). Any effects caused by PGs in the perfusate may thereby be obscured, and even it impossible to find out whether the PG levels are unchanged during hypoxia. Further

TABLE I. Vasoconstrictor responses to alveolar hypoxia I. Isolated lungs.

Expt no	Species	Blood flow (ml/min)	Baseline art. pressure (mmHg)	Peak rise in art. pressure during sequential hypoxic tests (mmHg) I	II	III	IV
1	rat	10	20	9	9	13	[illegible]
	rat	10	38	6	4	3	[illegible]
3	rat	10	--	8	8	12	[illegible]
4	rat	10	1	9	10	9	[illegible]
5	cat	40	14	7	7	5	
6	cat	15	15	13	10*	9*	

Hypoxic period 10 min.

until finally alveolar hypoxia no longer produces a pressor effect. Cat lungs on the hand can be perfused for several hours with increasing pressor responses (Duke and 1952)

Both patterns are, however, affected when the oxygenator and the bioassay system are included in the perfusion circuit: the period in which hypoxia induces responses is shortened, so that only a small number of such responses can be each pair of lungs. Also when the oxygenator and the bioassay circuit are included period with good pressor response, the responses rapidly decline. This was when rat lungs were used, most likely because the whole perfusate flow passed through oxygenator and bioassay circuit. With cat lungs only a small fraction (10 ml/min) of perfusate flow (152–240 ml/min) was led through the oxygenator and over the tissues. Nevertheless, also in this situation the number of pressor responses which be elicited was drastically reduced. This was tested separately in 2 cat lungs. In both the pressor response to hypoxia rapidly declined after opening to the bioassay-oxygenator circuit, as illustrated in Fig. 2.

During the total of 28 periods of hypoxia studied (Table I) *there was never any contraction of any of the 3 assay tissues* in spite of marked and fully reversible pressor responses. Even in the cases with 10 min periods of hypoxia the baseline tension of the assay remained stable. Fig. 3 shows effects of ventilation hypoxia in cat lungs. The bioassay is represented by the recording of RSS tension since this is the tissue most sensitive to

Periods of ventilation hypoxia after the pressor responses had disappeared did not produce any PG-like activity.

Lung tissue content of PGF was measured with radioimmunoassay. The 2 control frozen during ventilation with 5% CO_2 in air contained 114 and 82 ng PGF per g wet tissue, respectively. The 3 test lungs frozen during ventilation hypoxia and with pressor response contained 62, 80 and 69 ng PGF per g wet lung tissue.

Discussion

In this work we have mainly focused on alveolar hypoxia and PG-synthesis. Some additional observations relate, however, to other aspects of the pulmonary vasoconstrictor res

...th muscle, thus giving an increased pressor response to acute alveolar hypoxia ...nes and Hange 1975 Tyler et al. 1975, Weir et al 1976 a, Hales et al. 1977 ...75). This modulating action is rather small and has no relevance in elucidating ...m of the vasoconstrictor response to alveolar hypoxia.

...r results show that the pulmonary vasoconstrictor response to ventilation ...t mediated by stimulation of PG synthesis.

...m against PGF as the generous gift of Professor Lawrence Levine, Brandeis University USA. W are grateful to Dr John E. Pike, The Upjohn Company Kalamazoo, Mich USA PGs.

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more Said *et al* (1974) did not document any effect of calibrating doses of PGs on the assay tissues.

In the present experiments we showed effect of calibrating doses of 0.5–2 ng/ml of PGE and $PGF_{2\alpha}$ (Fig. 3) but we could not detect any change in tissue tension during short hypoxia. Effects caused by hypoxia per se was prevented by the oxygenator which maintained PO above 500 mmHg in the blood superfusing the assay tissues.

Although we found no trace of PGs in the blood stream we could not rule out the possibility that hypoxia induced PG synthesis in the lungs. PGs could then accumulate in lung tissue, locally influence vascular smooth muscle tone and subsequently be inactivated in the pulmonary circulation (Piper Vane and Wyllie 1970), without appearing in the venous effluent To test this hypothesis lung tissue content of PGs of the F-series was measured by radioimmunoassay The results of these measurements showed that in lungs exposed to alveolar hypoxia and with marked pressor responses, there was no increase in the tissue content of F prostaglandins. The tissue concentrations observed are similar to the low levels of $PGF_{2\alpha}$ reported earlier for rat lung tissue (Karim, Hillier and Devlin 1968).

Our results are thus in apparent conflict with the findings of Said and coworkers (1974) *In addition to those already mentioned there are several reasons, however* why their work is difficult to assess 1) They used a very low flow for perfusion of cat lungs (10 ml/min), 2) The basal level of P_{PA} was not presented and the reported responses to hypoxia were small (they considered a pressor response of 1 mmHg satisfying, but even this was not achieved in 8 of 32 expts.) 3) They used a perfusate of Krebs-dextran solution or a mixture of blood and Krebs-dextran Recently we have found that any perfusate containing dextran cause spontaneous PG-synthesis in isolated lungs. After perfusion for some time with dextran solution there is a progressive release of substances with PGE-like activity In this situation stimuli which are without any effect in lungs perfused without dextran, now release great amounts of PG-like activity in addition to the basal release (Wiberg, Scott and Vaage 1977 Vaage *et al* 1977 a)

In the present series of expts. an increased pulmonary synthesis of PGs with release and complete inactivation might theoretically escape detection. Several observations, however make such an event highly unlikely 1) Also PG-metabolites do to some extent cause contraction of the assay tissues (Crutchley and Piper 1975). 2) During pulmonary PG-synthesis unstable intermediates in PG-synthesis are released (Palmer *et al* 1973). These intermediates would partly have been converted to PGs of the E and F-series at the time when venous effluent blood reaches the assay tissues (Palmer *et al* 1973) 3) The radioimmunoassay results did not indicate any increase in PG-synthesis during hypoxia.

Kadowitz *et al* (1975) have reported that some synthesis of vasodilatory PGs is continuously going on in normal lungs. They found that indomethacin and meclofenamate, two structurally dissimilar inhibitors of PG-synthesis, increased pulmonary vascular resistance in dogs. These observations led to the suggestion that under resting conditions synthesis of vasodilatory PGs might be important for the maintenance of the pulmonary circulation in a dilated state. Such a background or basal production of PGs might as well play a role in setting the limits for any vasoconstrictor response to hypoxia. Inhibitors of
may act by preventing this modulating action of PGs with vasodilatory ef

…r smooth muscle, thus giving an increased pressor response to acute alveolar hypoxia …, Bertoni and Hauge 1975 Tyler *et al.* 1975 Weir *et al.* 1976 a, Hales *et al.* 1977 … *et al.* 1975). This modulating action is rather small and has no relevance in elucidating …echanism of the vasoconstrictor response to alveolar hypoxia.

…e present results show that the pulmonary vasoconstrictor response to ventilation …ich is not mediated by stimulation of PG synthesis.

…t antiserum against PGF… as the generous gift of Professor La…rence Levine, Brandeis University …m, Mass., USA. W… are grateful to Dr John E. Pike, The Upjohn Company Kalamazoo, Mich USA, …e gift of PGs.

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Effect of adenosine, adenosine analogues and drugs inhibiting adenosine inactivation on lipolysis in rat fat cells

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Abstract

м, B. B. *Effect of adenosine adenosine analogues and drugs inhibiting adenosine ctivation on lipolysis in rat fat cells.* Acta physiol. scand. 1978 *102* 191 198.

n suggested that adenosine may be physiologically important modulator of lipolysis. In the dy it was found that adenosine inhibited lipolysis stimulated by low (0 03 μM) concentrations of line (NA) Lipolysis stimulated by higher concentrations (0 3 and 3 μM) of NA as inhibited to ss or not at all. Theophylline (1 mM)-induced lipolysis was inhibited by adenosine (IC_{50} ~ 10μM). of theophylline-induced lipolysis was tested for several analogues of adenosine. Some N^6-sub-adenosine analogues and 2-Cl-adenosine ere more potent inhibitors. Adenine-nucleotides (ATP MP) were about equipotent with adenosine. Several adenosine analogues, including its breakdow were considerably less potent or ineffective. None of the analogues tested inhibited the action of . Dipyridamol, dilazep and papaverine, ich inhibit the uptake of adenosine into cells, caused light enhancement of the antilipolytic effect of adenosine. None of the analogues inhibited the adenosine. It is concluded that adenosine can inhibit lipolysis due to low "physiological" con-s of noradrenaline and of low concentration of theophylline via an action on receptor structure ll surface hich exhibits structural specificity

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ne is formed during incubation of isolated rat fat cells *in vitro* (Schwabe *et al.* 1973), e release of adenosine-like material from the isolated canine subcutaneous adipose *in situ* following sympathetic nerve stimulation was recently reported (Fredholm 1976). *in vivo* conditions adenosine is a good inhibitor of lipolysis induced by sympathetic stimulation (Fredholm and Sollevi 1977). Furthermore, dipyridamol, a known inhi- of adenosine inactivation (*e.g.* Schrader *et al.* 1972) was found to inhibit lipolysis ed by sympathetic nerve stimulation (Fredholm and Sollevi 1977). These findings are tible with the opinion that adenosine might act as a physiologically important mo- r of lipolysis.

n interpretation of the results is, however complicated by the published findings of

viations: ATP-adenosine 5'-triphosphate; ADP-adenosine 5'-diphosphate AMP-adenosine 5' phosphate; cyclic AMP-adenosine 3',5'-cyclic monophosphate, AMP (NH)pp-adenylyl-imidodiphos- , AOPCP-α, β-methyleneadenosine 5'-diphosphate, EHNA-erythro-9-(2-hydroxy-3-nonyl) adenine.

adenosine effects in isolated rat adipocytes. There is complete agreement in the l that adenosine and several analogs including the phenylisopropylderivative are inhibitors of cyclic AMP accumulation induced by catecholamines (Fain 1973 a, S *et al* 1973 Stock and Prilop 1974 Hjemdahl and Fredholm 1976 a). On the oth adenosine is remarkably ineffective as an inhibitor of lipolysis (Fain 1973 a, Schw 1973 Hjemdahl and Fredholm 1976 a) As suggested by Fain (1973 b) this appa crepancy between the effects on cyclic AMP and the effects on lipolysis might be e if the pool of cyclic AMP involved in turning on lipolysis is small and not in eq with the bulk of the cyclic AMP in the cells. In isolated rat fat cells we recently rep the relationship between cyclic AMP and lipolysis (Hjemdahl and Fredholm 197 found that elevations of cyclic AMP by only 2–3 pmol/10^6 cells was necessary t half maximal stimulation of lipolysis. Since the basal cyclic AMP content in o averaged 4 pmol/10^6 cells this represents only a 50 per cent increase in cyclic AMP S we recently found that under *in situ* conditions maximal lipolysis induced by sy nerve stimulation was associated with a 50 increase in tissue cyclic AMP conte holm, Belfrage and Blaschke 1977)

The effectiveness of adenosine as on antilipolytic agent *in vivo* might therefore l the fact that the cyclic AMP levels *in vivo* are low Conversely the relative ineffect adenosine as on antilipolytic agent *in vitro* may be due to the fact that the *in vit* commonly employ maximally effective concentrations of catecholamines which p several fold increases in cyclic AMP

From the arguments summarized above it was considered important to reco effect of adenosine on lipolysis caused by submaximal concentrations of lipoly For this purpose low concentrations of noradrenaline and low concentrations of the were chosen. In addition we have studied the effect of a number of analogues of t and of some substances known to interfere with the disposition of adenosine.

Materials and Methods

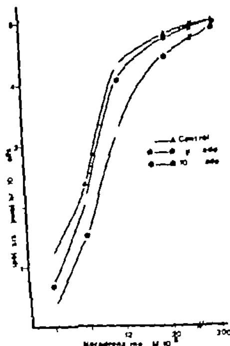
Experimental

Male Sprague Dawley rats (Anticimex) weighing 160–220 g were used. Adipocytes were isol cubated according to Rodbell (1964) as previously described (Fredholm and Hjemdahl 1976 a) cell concentration was 30–80 10^3 cells/ml The cells were preincubated for 10 min with the respe Lipolysis was initiated by the addition of theophylline (final concentration 0.1 mM) or no (f nal concentration 0.03–3 μM). I the latter case 10 μg ascorbic acid was included per ml of the buffer 1 h after the addition of the lipolytic drug 0.2 ml medium was deproteinized by ZnSO precipitation and glycerol determined accordi g t La rell and T bbling (1966)

Cyclic AMP was determined by a compet tive bind ng assay (Brown *et al* 1971) in 1 0 ab incubations, withdrawn 5 ml after the addition f the lipolytic drug pipetted into 0.5 ml ice- perchloric acid. After neutralization with 4 M KOH and 1 M Tris-base (pH 7 7 8), cyclic AMP mined in 50 μl aliquots of the supernatant. 85–100 per cent of the binding material was remov phosphodiesterase (Boehringer Mannheim). Purification of the samples on Dowex 50 4 (200- columns was not necessary since the values obtained were identical with those found without p after correction for losses during purification.

Chemicals

Crude bacterial collagenase (from Cl. histolyticum) was obta ned from Worthington (Freeh Theophylline was used as the ethylenediamine salt (Oxyphylline® Astra, Södertälje, Swede serum albumin (Fraction V) *l*-Noradrenaline hydrochloride, ATP ADP AMP Adenosine 8-bromo-adenosine, 2-chloro-adenosine were btained from Sigma (St. Lou USA),



1. The effect of adenosine on the lipolytic ... to noradrenaline. Mean of 2–4 determinations ... 2 separate expts. (expt. 1. 67 000 cells/ml, ... 42 000 cells/ml). The cells were preincubated ... µg ascorbic acid and adenosine at the concentrations indicated in the buffer for 10 min before ... of noradrenaline.

...posinathine from Merck (Darmstadt, BRD), inosine and adenylyl-imidodiphosphate from Boehringer ...heim, BRD), α,β-methyleneadenosine 5'-diphosphate (AOPCP) from PL Biochemicals (Milwaukee, ...) N[6]-phenylisopropyladenosine (PIA) and N[6]-cyclohexylalkyl-adenosine were kind gifts from Dr. H. ..., Boehringer Mannheim BRD. N[6]-(Δ₂-isopentyl)-adenosine was kind gift of Dr. Bruce Hacker ...y, N.Y. USA), and was dissolved in dimethylsulfoxide before use. N[6]-(3-hydroxy-3-methyl-butyl) ...osine and N[6]-butylidene-adenosine were kind gifts from Dr. H. Vorbrüggen, Schering AG (Berlin, ...). The adenosine deaminase inhibitor erythro 9 (2-hydroxy-3-nonyl) adenine hydrochloride (EHNA) ...h from Wellcome Res. Labs. (Research Triangle Park, USA). Dilazep was gift from Dr. N. Brock, ... Werke (Brackwede, BRD). Dipyridamol was gift from Boehringer Ingelheim (BRD). Papaverine ...rochloride was obtained from ACO (Sweden). 3-isobutyl-1-methyl xanthine was obtained from Aldrich ...mical (Milwaukee, USA). Other chemicals were obtained from ordinary commercial sources.

Results

...agreement with previous findings (see Introduction) adenosine did not cause any significant inhibition of lipolysis induced by 3 µM NA. Lipolysis induced by 0.3 µM NA was ...hibited by only 10% at 100 µM concentrations, while lipolysis induced by 0.03 µM NA ...s inhibited more than 50% already at 1 µM adenosine in the medium (Fig. 1 a). Very ...milar results were obtained with phenylisopropyladenosine (not shown).

Corresponding cyclic AMP values are shown in Table I. Already at 0.3 µM noradrenaline ...a essentially maximal cyclic AMP response was obtained. In the presence of adenosine ...(10 µM) noradrenaline caused small, statistically not significant, increases in cyclic AMP ...n agreement with previous results (Fain 1973 a, Schwabe *et al.* 1973, Hjemdahl and Fred...holm 1976 a).

The antilipolytic effect of adenosine was more pronounced at low concentrations of theo-

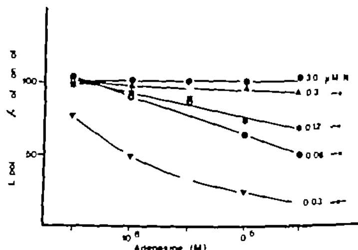


Fig. 1b. Inhibition of lipolysis by adenosine Data were obtained from the same 2 expts. Results ex pressed as per cent of the lipolysis induced by the corresponding concentration of noradrenaline in bsence of adenosine. Statistical hypothesis were tested by student s t-test. When lipolysis as activated 0 03 µM NA ignificant inhibition (p<0.01 or better) was obtained already ith 0.3 µM adenosine 0 06 µM NA significant inhibition (p<0.001) with 1 µM adenosine nd higher by 0 12 µM NA signif inhibition (p<0 05) with 3 µM adenosine by 0.3 µM NA significant inhibition (p<0 01) of lipolysis required 30 µM adenosine.

phylline than at high (Fig. 2) The same was found for another methylxanthine, isobutyl methylxanthine (MIX) (Fig. 2).

The effect of adenosine and several adenosine analogues on lipolysis stimulated by 1 mM theophylline is shown in Table II It can be seen from this table that the N⁶-substituted adenosine analogues namely phenylisopropyl-adenosine, cyclohexylallyl adenosine and hydroxy-3-methyl butyl adenosine as well as 2-Cl-adenosine are more potent than adenosine A fourth N -substituted analog, ∇2 isopentyl adenosine is slightly less potent and the butyldiene-adenosine, is practically devoid of activity 8-bromoadenosine, 2-deoxyadenosine, inosine, adenine, and EHNA similarly have a very low activity AMP and ADP are roughly equipotent with adenosine, while the ADP analog AOPCP is much less active. ATP and its analogue AMP(NH)PP are less active than adenosine.

Thus, some adenosine analogues are more potent than the parent drug. One possible explanation for this finding is that they are less readily taken up and/or metabolized the

TABLE I Cyclic AMP in fat cells treated with noradrenaline with and without adenosine and theophylline Results are shown as means ± S_x The number f incubation is shown within parenthesis

	Cyclic AMP pmol 10^{-6} cells/5 min		
	No adenosine	10 µM denosine	
Control	4 0±0.9 (18)	4.2±2.2 (10)	N.S.
Noradrenaline			
0.03 µM	5.0±1 7 (17)	4 6±2.4 (10)	N.S.
0.3 µM	10.0±1.3 (18)	5.1±2.2 (7)	p<0.001
3 0	11.5±1 4 (17)	5.6±3.2 (8)	p<0 001
30	9 0±1.2	—	
Noradrenaline 3 µM + Theophylline 1 mM	259±30 (24)	41±15 (6)	p<0 001

. Inhibition by adenosine, analogues and related compounds of theophylline (1 mM) induced lipolysis. IC_{50} values were determined from the linear portions of the log-dose response curves ob- tained from quadruplicate determinations in at least tw separate experiments.

	IC_{50} (μM)	Lipolysis in of control at 10^{-4} M
[illegible]	8 5	44
[illegible]		
	10	53
	9	44
	24	72
DPP	35	76
P	—	106
[illegible]		
[illegible]isopropyl)adenosine	0.8	7
[illegible]adenosine	0 9	8
[illegible]hydroxy-3-methylbuty)-		
[illegible]	4	17
[illegible]clohexyl)ethyl)-adenosine	8 5	47
[illegible]adenosine	20	64
[illegible]-adenosine	—	74[1]
[illegible]-adenosine	—	90
[illegible]	—	97
[illegible]adenosine	—	86
[illegible]	—	103
A	—	84
drugs		
[illegible]	—	105
[illegible]verine	—	106
[illegible]damol	—	102

[1] Partial inhibition (32 %) as produced at 3 μM.

[...]nosine. In order to test this possibility the 3 inhibitors of adenosine degradation, di[...]idamol, dilazep and papaverine were used. The results show that these drugs cause a [...]ght enhancement of the antilipolytic effect of adenosine, but the shift is a minor one [...]g. 3).

Another group of analogues appeared to be much less potent than the parent drug. The [...]sibility existed that these drugs were partial agonists and hence could act to block the [...]tions of adenosine. This possibility was tested. However none of the drugs could inhibit [...] antilipolytic effect of 10 μM adenosine. Moreover 2-deoxyadenosine, which was virtually [...]effective as an antilipolytic drug when used alone, inhibited lipolysis in the presence of [...]enosine by 50 per cent at a concentration of 50 μM.

Discussion

[...]he present results demonstrate that adenosine is antilipolytic *in vitro* provided that the [...]oncentration of the lipolytic drug is kept low. With the lowest dose of noradrenaline (0.03

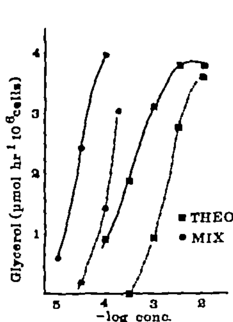


Fig. 2

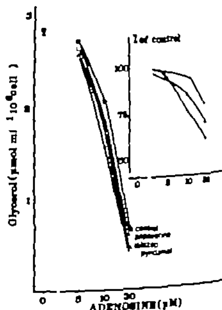


Fig. 3

Fig. 2. Lipolytic effect of theophylline (■) and 3-isobutyl-1-methylxanthine, MIX, (●) in the pr… (broken line) and …bsence (intact line) of adenosine 10 µM The cells (46 000/ml) were preincub… buffer for 10 min before the addition …f the methylxa…thines and adenosine. Means of triplicate deter… tions.

Fig. 3 Effect increasing doses of adenosine in the presence of 30 µM papaverine (■), 30 µM dilaz… and 3 µM dipyridamol (▽) on lipolysis induced by 1 mM theophylline. Mean of duplicate determ… from …separat… expts. (expt. 1 46 000 cells/ml, expt 2, 42 000 cells/ml) Insert lipolysis in the pres… papaverine, dilazep and dipyridamol in … of l polysis in the presence of adenosine alone.

µM) or with low doses of methylxanthines (0.3 mM theophylline, 0 03 mM MIX) ade… caused an essentially complete inhibition in a concentration of 10 µM

When higher doses of the methylxanthines were used the antilipolyti… effect of ade… decreased as would be expected from an essentially competitive inhibition. On the… hand when high concentrations of noradrenaline (1 µM or above) were used to sti… lipolysis even very high concentrations of adenosine did not inhibit lipolysis. This… agreement with the results of Fain *et al* (1972) and of Stock and Prilop (1974). On the… hand, Ebert and Schwabe (1973) found a decreased maximal lipolytic response to… drenaline by adenosine. We have no ready explanation for this discrepancy

It has been shown repeatedly that adenosine inhibits noradrenaline induced cyclic… elevations in fat cells to a much larger degree than lipolysis (Schwabe *et al* 1973, Fai… 1972, Stock and Prilop 1974 Hjemdahl and Fredholm 1976 a). This might be inter… as evidence for a dissociation between cyclic AMP and lipolysis (*e.g* Stock and Prilop… However the relationship between drug induced cyclic AMP elevations and drug-in… lipolysis is not linear Very small elevations of cyclic AMP are necessary to produce… tially maximal lipolysis (Hjemdahl and Fredholm 1976 a) It is possible that the very… increases in cyclic AMP due to noradrenaline in the presence of adenosine (Schwabe… 1973 Hjemdahl and Fredholm 1976 a, Table I present study) is sufficient to expla… induced, essentially maximal, lipolysis. It is generally assumed …cyclic AMP inc…

sis via activation of protein kinase that activates a hormone-sensitive lipase (see Steinet al. 1976). Thus, cyclic AMP bound to the regulatory subunit of protein kinase may a functionally important part of the nucleotide. In agreement with such a view it was dy found that the bound form of cyclic nucleotide changes over a much smaller range the free, possibly less important form of the nucleotide (Terasaki and Brooker 1977). ibitors of cellular adenosine uptake such as dipyridamol, dilazep and papaverine do nhibit the actions of adenosine on lipolysis and cyclic AMP accumulation (Fain et al. Ebert and Schwabe 1973, present data). If anything a slight potentiation of the action lenosine was seen. This clearly suggests that adenosine acts on the outer surface of the These findings also tend to rule out inhibition of protein kinase (e.g. Kariya and Field) as an important mechanism in the antilipolytic action of adenosine.

he bulk of the evidence thus suggests the existence of a surface adenosine-receptor pled to the fat cell adenylate cyclase by some unknown mechanism. In order to study the ctural requirements at this receptor site a number of analogues were tested. Of the rally occurring analogues AMP and ADP were approximately equipotent with adeno- ATP was less potent, while inosine and adenine were essentially devoid of activity restingly the two "stable" nucleotide analogues AMP(NH)PP and AOPCP were conrably less active than their unstable natural counterparts, suggesting that breakdown to nosine is essential for activity. The finding that breakdown products of adenosine are ntially devoid of activity is in agreement with earlier results (Fain et al. 1972, Ebert and wabe 1973).

n agreement with earlier findings we found that N⁶-phenylisopropyladenosine was more tent than its parent compound (Fain et al. 1972, Ebert and Schwabe 1973). 2-chloronosine was also almost ten times as potent as adenosine. Since these compound are istant to the action of adenosine deaminase the high activity could be due to a lower rate degradation. However adenosine in the presence of EHNA, a very potent inhibitor of enosine deaminase, or in the presence of either of three inhibitors of adenosine uptake, s still considerably less potent than 2-chloradenosine and PIA, rendering this possibility s likely. Furthermore, adenosine deaminase activity in fat cells is low (Fredholm, unblished). Thus, certain adenosine analogues may have a higher affinity than adenosine the proposed receptor while other analogues have a lower affinity and/or lower intrinsic tivity. The latter possibility was particularly interesting since a partial agonist with low trinsic activity may be used as an inhibitor. Therefore the analogues with low activity were sted in combination with adenosine. However, none of these compounds were found to gnificantly antagonize the effect of adenosine. It is reasonable to assume therefore that eir low biological activity is due to low affinity to the receptor rather than to low intrinsic ctivity.

Under physiological conditions noradrenaline appears to be lipolytic in very low concentrations. Thus, when noradrenaline is given by i. infusion essentially maximal rates of lipolysis are seen when calculated plasma concentrations are as low as 0.02 μM (Hjemdahl and Fredholm 1976 b). Also during nerve stimulation the effective noradrenaline concentrations are quite low since most fat cells are not in direct contact with nerve terminals (Fredholm 1970, Belfrage et al. 1977). Therefore the present results showing an antilipolytic

effect of adenosine provided that the concentration of noradrenaline is in the physiological range offers support for the possibility that adenosine may play a physiological role as a modulator of lipolysis (Fredholm 1976, Fredholm and Sollevi 1977).

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Acta physiol. scand. 1978. 102. 199–204

From the Department of Clinical Physiology Karolinska Institutet at Huddinge Hospital, Sweden

Prostaglandin-mediated inhibition of noradrenaline release· II. Dual mechanism behind its frequency-dependence

By

ÅKE WENNMALM

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Abstract

…NMALM, Å. *Prostaglandin-mediated inhibition of noradrenaline release II. Dual mechanism behind its frequency-dependence* Acta physiol. scand. 1978 *102* 199–204

…pathetically innervated, isolated rabbit hearts were perfused according to Langendorff and the nerves …stimulated at 2, 5 or 10 Hz by equally long trains of pulses. The outflows of prostaglandin-like sub…ces (PLS) and of noradrenaline (NA), induced by the nerve stimulations, were followed. The sensitivity …he process of NA release to exogenous PGE_1 (2–6 10^{-9} M) at 2, 5 and 10 Hz was analyzed. The outflow …PLS …as found to be frequency-dependent, being greater at 2 Hz than at higher discharge rates, while …outflow of NA …as similar at the different frequencies. The inhibitory action of PGE_1 on the NA …se process …as more pronounced at 2 than at 10 Hz. It is concluded tha… the frequency-dependence of …ndogenous PGE-mediated inhibition of the release of NA from discharging sympathetic nerves is …ed on … independent, frequency-related mechanisms: a) … higher synthesis rate of PLS/impulse, and b) …ore pronounced sensitivity of the process of NA release, at low compared to higher impulse frequencies.

…dogenous prostaglandins of the E series (PGE) have an inhibitory action on the process …release of sympathetic neurotransmitter (noradrenaline, NA) from depolarising adrenergic …rves in perfused organs from various species (Samuelsson and Wennmalm 1971 Hed…vist, Stjärne and Wennmalm 1971 Swedin 1971 Chanh, Junstad and Wennmalm 1972, …errera, Moncada and Vane 1973, Starke and Montel 1973). There is evidence that this …ction of endogenous PGE is present also in intact animals (Stjärne 1971 Junstad and Wenn…malm 1972, Fredholm and Hedqvist 1975). The mechanism by which PGE operates to …depress the release of NA from depolarising nerves is incompletely known. Hedqvist (1970) …demonstrated that the inhibitory action elicited by exogenous PGE_1 on NA release from …he perfused stimulated cat spleen was counteracted by increased calcium concentrations …n the perfusing medium, and this observation was confirmed in the guinea pig vas deferens …(Stjärne 1973 a). Later the capacity of endogenous PG to counteract the release of NA …as demonstrated to relate inversely to the depolarising frequency in the nerves (Junstad …and Wennmalm 1973, Stjärne 1973 b). The latter observation, suggesting that the inhibitory …action of endogenous PGE is most pronounced …t low depolarising frequencies (1 2 Hz),

supports the "physiological" significance of the PGE mediated inhibition. The investigation was therefore undertaken to study the mechanism behind this f dependence. The method chosen was to investigate, first, the outflow of PLS (prostaglandin-like substances) from the heart at different impulse frequencies in its sympathetic and second the sensitivity of the NA release process to PGE at different impulse frequencies.

Methods

a. *Rabbit heart perfusion*

Rabbits of mixed strains and sexes were used for the study. The weight of the animals varied from kg. After a blow on the head, the animal was exsanguinated by cutting the left carotid artery. The was dissected free with its bilateral sympathetic nerve supply kept intact (Huković and Muscholl). It was subsequently transferred to the perfusion apparatus, where it was perfused according to Langendorff with Tyrode solution of the following composition (in mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, Mg $NaHCO_3$ 1, NaH_2PO_4 0.4, glucose 5.6. The solution was aerated continuously with 5% CO_2 in pH of the solution was 7.4–7.5 and its temperature was kept at 37°C. The sympathetic nerves to the heart were pulled through plastic tubes with circumferential platinum rings serving as electrodes. The electrodes were connected to a Grass Model S88 stimulator. The nerves were stimulated with trains of 1 000 pulses, 25 V in height, and of 1 ms duration, delivered at 2, 5 or 10 Hz.

In one series the effect of varying the frequency of the sympathetic nerve stimulation on the outflow of PLS from the heart was analysed. Each heart was stimulated three times, at 2, 5 and 10 Hz respectively, the stimulations being separated by a resting interval of 15 min. The venous effluent from the heart was collected from the beginning of each stimulation and for the following 10 min (t Hz), 5 min (at or 4 min (t 10 Hz). The sequence of stimulation frequencies was varied between the expts. so that in one third of the series the expts. were started at 2 Hz, in one third at 5 Hz and in one third at 10 Hz.

In another series the capacity of exogenous PGE to inhibit the outflow of NA from the heart at different frequencies of sympathetic nerve stimulation, was studied. The hearts, dissected and perfused as above, were stimulated twice, with a 15 min interval, at either 2, 5 or 10 Hz, with trains of 1 000 PGE was infused through a cannula above the aorta into the solution perfusing the heart, from of the first effluent collection period until the end of the expt. The final concentration range of PGE –6 10^{-9} M. NA in the effluent was analysed using the trihydroxyindole method.

b. *Analysis of PLS*

The venous effluent from the heart was collected on ice and within 1 h after the collection PLS was on 7 g columns of Amberlite XAD-2. PLS was eluted with ethanol. After evaporation to dryness residue was dissolved in 1 ml of saline. The biological activity of the dissolved residue was tested known amounts of PGE_2 on the superfused rat stomach strip (Vane 1957). The assay organ was 5 ml organ bath containing Tyrode solution of the composition given above, to which was added amine ($7\times10^{-}$ M), propranolol (8 10^{-7} M), atropine (10^{-} M), methysergide (6 10^{-} M), phenhydramine (7×10^{-7} M), in order to block activity in the strip due to the presence in the lipid of noradrenaline, acetylcholine, serotonine and histamine. Usually 1/2 of the lipid extract from the was tested on the stomach strip. Since the assay organ was sensitive to prostaglandin of the E- amounts of 1 2 ng, an outflow of prostaglandin-like substances in the effluent during the perfusion period of less than 5 ng could be detected. Each lipid extract was tested twice and the intra-assay variation in the samples t repeated assays was usually less than 15 %. The recovery of the prostaglandin extraction procedure was checked in every extraction series by the use of external standards, and the given for outflows of PLS have been corrected using the actual recovery (72±5%).

c. *Calculations*

The outflow of PLS was calculated as follows. After bioassay against PGE_2 and volume correction PLS content of a sample was corrected for the actual recovery of PGE_2, estimated using the external standard. The outflow of PLS is presented as the mean ± S.E. of the outflow of PLS at 10 Hz, respectively. The outflow figures are also presented as outflow of PLS/min, by dividing actual total outflow by the duration of the effluent collection period. Finally the outflow figures as outflow of PLS/impulse, by subtracting from the total outflow the estimated basal outflow the respective effluent collection periods.

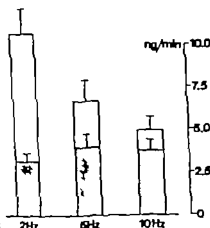


Fig. 1

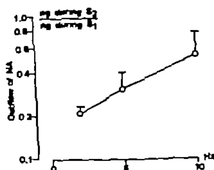


Fig. 2

. Outflow of PLS from the sympathetically stimulated rabbit heart. The total height of the columns ...tes the outflows of PLS during 1 000 pulse trains of nerve stimulation at 2 Hz, 5 Hz, or 10 Hz (left ...ate), and the striped columns indicate the outflow of PLS/min during the respective frequencies of stimulation (right ordinate). All values are presented as mean ± S.E.

2. Effect of PGE_1 (2–6 10^{-8} M) on the outflow of NA from the isolated rabbit heart, sympathetically ...lated at 2 Hz, 5 Hz, or 10 Hz. The values given represent the ratio between the outflows of NA during consecutive nerve stimulations (1 000 pulses), delivered at the frequencies indicated. Values given as ± S.E.

...e capacity of exogenous PGE_1 t inhibit the release of NA during nerve stimulation is expressed ...e ratio between the NA in the effluent during the second (PGE_1 present) and the first periods of stimulation and effluent collection.

Results

Outflow of PLS at different frequencies

...mulation of the sympathetic nerves to the heart t 2 Hz with trains of 1 000 pulses elicited ...increase in heart rate (29 ± 4 beats/min) and contractile force. An outflow of NA appeared ...he venous effluent, amounting to 179 ± 30 ng, during the stimulation period. The outflow ...PLS was markedly increased, corresponding to almost 30 ng of PGE_2 (Fig. 1) during the ...nin effluent collection period. After the basal outflow of PLS had been subtracted, the ...utflow of PLS induced by the 2 Hz sympathetic nerve stimulation corresponded to 19 ± 4 ... PGE_2/imp.

At 5 Hz the mechanical response of the heart to trains of 1 000 pulses was more pro...unced, the heart rate being increased by 51 ± 5 beats/min. The outflow of NA was, ...wever of the same magnitude as that obtained at 2 Hz (183 ± 39 ng). The total outflow ... PLS at 5 Hz was about 2/3 of that obtained t 2 Hz (Fig. 1). Estimated as outflow ... PLS/min it was, however the same as that at 2 Hz. The net outflow of PLS at 5 Hz ...otal-basal) corresponded to 12 ± 3 pg PGE_2/imp.

The 10 Hz sympathetic nerve stimulation elicited the most marked mechanical response of the heart inducing an increase in heart rate of 70 ± 9 beats/min. The overflow of NA was slightly but insignificantly higher (212 ± 52 ng) than at 2 Hz or 5 Hz. The total outflow of PLS decreased further as compared to 2 Hz and 5 Hz. However expressed in ng PGE/min, the outflow of PLS at 10 Hz was of the same order as at 2 or 5 Hz (Fig. 1). The outflow of PLS at 10 Hz (total-basal) corresponded to 11 ± 3 pg PGE_1/imp.

b *Sensitivity of NA release to PGE_1 at different frequencies*

Stimulation of the sympathetic nerves at 2 Hz (1 000 pulses) was followed by an overflow of NA of 710 ± 295 ng heart rate increased 30 ± 6 % (41 ± 8 beats/min). A second stimulation at 2 Hz in the presence of PGE (2–6 10^{-8} M) elicited a release of NA amounting to only about 20 (155 ± 75 ng) of that obtained during the first nerve stimulation (Fig 2) The chronotropic response was unaffected, or even slightly increased (40 ± 7 46 ± 6 beats/min) during the second nerve stimulation, the ratio between the chronotropic responses during the second and first nerve stimulation being 1.20 ± 0.07

At 5 Hz the overflow of NA during the first nerve stimulation was 495 ± 90 ng heart rate increased 42 ± 14 (51 ± 11 beats/min). PGE (2–3 10^{-8} M) inhibited the overflow of NA during the second nerve stimulation at 5 Hz to about 30 % of the control (Fig 2). The chronotropic response (43 ± 15 – 51 ± 14 beats/min) was unaffected by the presence of PGE during the second nerve stimulation.

When the sympathetic nerves were stimulated at 10 Hz, 1 040 ± 475 ng of NA appeared in the effluent heart rate increased 57 ± 3 % (84 ± 4 beats/min) PGE (2–4 10^{-8} M) inhibited the overflow of NA during the second 10 Hz nerve stimulation to about of the control (Fig. 2) The chronotropic response (48 ± 5 % – 64 ± 4 beats/min) was slightly smaller in the presence of PGE during the second nerve stimulation

Discussion

In the present study equally long trains of pulses stimulating the sympathetic nerve supply to the isolated rabbit heart elicited the same overflow of NA into the venous effluent when delivered at 2, 5 or 10 Hz. In contrast the venous outflow of PLS varied with the frequency, being greater during the 2 Hz stimulation compared to 5 Hz and 10 Hz. The larger outflow of PLS at 2 Hz was apparently due to the longer effluent collection time at this frequency compared to 5 or 10 Hz, when expressed in relation to time there was no difference between the outflow figures of PLS at the different frequencies. The mechanism leading to the frequency-dependence is not obvious. It might be argued that since the different stimulations, at various frequencies but of the same number of pulses, gave rise to similar NA overflows, a stoechiometric relation would decide the amount of PLS synthesised per mole NA. However the actual concentration of NA at the PLS synthesis stimulation sites should have been considerably higher at the 10 than at the 2 Hz stimulations in the present experiments and consequently PLS synthesis should have been more stimulated at the higher frequency. More plausible, then, are either of the alternatives that the PLS synthesis stimulation induced by sympathetic nerve stimulation is fully developed already at 2 H nd that an increase

rate does not further elevate the degree of PLS synthesis stimulation, or alternatively that there is a delay in the onset of PLS synthesis stimulation following nerve stimulation ...day that affects short stimulation periods relatively more than longer ones.

...nsitivity of the NA release to exogenous PGE varied also with the frequency of ... in the present study being considerably greater at 2 than at 10 Hz. Hedqvist ...d Stjärne (1973 a) reported that PGE, when inhibiting the release of NA, acts by ...g with the availability of Ca... Furthermore, in the same paper Stjärne demon... curvilinear relationship between the inhibitory effect of PGE and the calcium ...tion in the medium on the neuronal NA release. In the light of these results the ...data, showing less inhibitory effect of PGE at increased discharge rates, may be ...ted as follows: increased depolarization frequency in the sympathetic nerves elicits ...eased neuronal concentration of Ca^{++} available for the NA release. At a certain ...ration of PGE the inhibitory action of this drug on the NA release process is less ...ced at higher than at lower Ca^{++} concentrations. The more pronounced inhibitory ...f PGE at 2 Hz compared to 10 Hz would thus be expected.

...nclusion, the frequency-dependence of the inhibition of NA release from depolarising ...hetic nerves, induced by endogenous PGE, seems to be based on 2 independently ...frequency-related mechanisms, a) a greater outflow of PGE per nerve impulse at low ... high discharge rates, and b) an increased sensitivity of the NA release process to the ...ory action of PGE at low compared to high impulse frequencies.

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Acta physiol. scand. 1978. 102. 205–217

From the Institute of Zoophysiology University of Oslo, Norway

The spatial distribution of odour induced potentials in the olfactory bulb of char and trout (Salmonidae)

By

GEORG THOMMESEN

Received 12 July 1977

Abstract

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…olar DC-recordings were made of the gross responses from the olfactory bulb of char (*Salvelinus* …sp. *Salmo alpinus* L.) and trout (*Salmo trutta* L.) during stimulation with different odours. The …e features studied were the magnitude and polarity of the slow potential shift, the amplitude of …luced waves and their asymmetrical waveform. Amino acids elicited the largest responses in the …part of the bulb. Water containing "crude fish odour" caused the largest responses in the rostral …edial parts. The results demonstrate odour specific differences in the localization of the bulb re…s and the separate origin of the slow potential and the induced waves.

…ords: Odour stimulation, response specificity, spatial distribution, olfactory bulb, teleost

…vertebrate olfactory organ serves several purposes, including seeking for food and …specific communication. In salmonid fishes the intraspecific communication may …ido pheromone detection important for the homing behaviour of the spawners (Nordeng …l Solomon 1973).

…he adequate stimulants for the salmonid olfactory organ are predominantly amino acids …Herbn and Sutterlin 1971) and related compounds and yet unidentified components of …fish skin mucus (Døving, Enger and Nordeng 1973). Such components are suspected to …e as pheromones (Døving, Nordeng and Oakley 1974).

…Anatomical investigations on the teleost olfactory system show a diversity in the central …jections of olfactory bulb onto various parts of the brain (Sheldon 1912, Holmgren 1920). …atomical and physiological studies on the olfactory tract of the burbot show that it is …divided in bundles with different spectra of fiber diameters and conduction velocities …ving and Gemne 1965).

From electrophysiological and anatomical work on mammals it is known that the ol…ctory bulb is functionally subdivided. Information on different odours is treated by …ferent, if not necessarily exclusive parts of the bulb (Adrian 1951) involving partially …parated populations of secondary neurons (Pinching and Døving 1974). It is therefore

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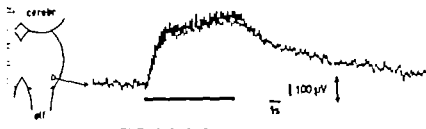
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Vane, J. R., A sensitive method for assay of 5-hydroxytryptamine. *Brit. J. Pharmacol.* 1957, 12



The general response characteristics illustrated by DC recording of the response to 10^{-4} M of ... The recording was made from the surface of the rostrolateral corner of the left olfactory bulb. The marker pen trace (bottom) shows the switching of the stimulation valve. The thick bar above the real stimulation period. Note the asymmetry of the induced waves

... consecutively. The order of the stimuli was varied at random. To avoid adaptation stimulation ... were at least 30 ... or as long as any sign of the previous response remained. Stimulation time was ... off manually and adjusted for the time lag present due to the construction of the stimulation device.

... of responses

... shifts were measured as the mean deflection of the tracing at 3–4 ... after the onset of the ... Due to the general instability of the DC recording the measurements of the DC deflections are ... within ± 30 µV. The AC-responses were measured as the mean amplitude of 3 ... recording, from ... to 5 ... after the onset of the response, by means of ... polar planimeter. The polarity of any asymmetry ... recording was noted. As ... control on possible differences in physical conditions such as ... variable ... of the olfactory bulb surface and shunting effects, the spontaneous activity previous to each ... has been evaluated by the same method as the AC-responses. The measurements of the AC signals ... standard deviation of about ± 10 µV as judged from ... great number of quantifications of the spontaneous activity.

Results

... response characteristics

... bulb surface responses to olfactory stimulation had two main components as previously ... by Ottoson (1959 a and b), a sustained component or DC-potential shift and ... oscillations hereafter referred to as "induced waves" (Adrian 1950). An example of ... response with the typical characteristics is shown in Fig. 1. With the stimulation system ... in these experiments the latency could not be determined. The response increased grad... during the first 2–3 s of stimulation. At this time a breaking point was often seen, after ... the response stabilized and persisted as long as the stimulus was maintained for up to ... several tenths of seconds. When the stimulus was turned off the response declined roughly ... exponentially with ... halftime of 3–10 s.

... typical DC potential shifts were monophasic deflections of the baseline, with a magni... ranging from ... 250 to −1 000 µV.

... some recordings the DC shift was disturbed by mechanical artifacts due to the switching of the stimula... valve and the contemporary transient flow change. These artifacts were short lasting, usually positive ... deflections of up to 100 µV. Occurring simultaneously with the manual stimulation marking, they ... easily detectable and did not interfere with the true responses. Positive deflections simultaneous with ... the stimulation and ... with rapid onset and ending were also seen. These deflections did not seem to be ... related to stimulus concentration. Similar reactions could be artificially produced by stimuli diluted in ... distilled water instead of the usual aquarium water. They disappeared when the electrode was placed

likely that the bulbo-cerebral connections in the fish pointed out by Sheldon (19.. Holmgren (1920) are reflected in the functional organization of the fish olfactory bulb

The aim of the present study was to find the spatial differentiation of the responses of the bulb to various odours. In this article the odour specific differences in the responses are described by examples of the responses to stimulation with some amino acids and "fish odour" samples. A preliminary report of this work has been made (Thommesen

Material and Methods

The material consists of experimental series, one on the brown trout (*Salmo trutta* L.) and one on arctic char (*Salvelinus alpinus* syn. *Salmo alpinus* L.). The present study is based upon recordings from trouts and 18 chars. The trouts, weighing about 100 g, were caught by an electrical fishing apparatus in a local brook in Oslo. 16 of the chars were hatched in 1970 as offspring of a few parents caught simultaneously at their common spawning site in lake Lona in western Norway (Nordeng, personal communication). These chars were therefore presumed to be as genetically homogeneous as could be expected in an interbreeding population in nature. At the time of experiment they were about 6 years old and weighed about 300 g. Two of the chars had been caught by net in the neighbouring lake Vangsvatn.

The fish was paralyzed with Tubocurari (Nyco) 1 mg tubocurarin-chloride i.m. per 100 g. The paralyzed fish was wrapped in a wet sponge and secured in a Plexiglass form by steel rods and claws of a headholder. The gills were perfused with aquarium fresh water (Oslo city water) at 5–... dorsal aspect of the olfactory bulbs was exposed. Each bulb was approximately 1 mm across in the ... 1.5 mm in the chars.

Recording. The potentials were picked up by glass capillaries filled with a physiological salt solution ... electrodes were inserted in electrode holders containing sintered Ag/AgCl. Electrode tip diameter from 20–100 μm. The potential changes were fed to preamplifiers with frequency response DC–1 50 Hz and monitored on a pen recorder. All recordings were monopolar, and the preparation was ... ed through the indifferent electrode only, which was placed on the chondrocranium just ... opening in the scull.

Stimulation

The stimuli were presented from a polyethylene pipette through a continuous flow of aquarium water ... the ipsilateral anterior naris and controlled by 3-way stopcock. Nasal water flow was kept at ...

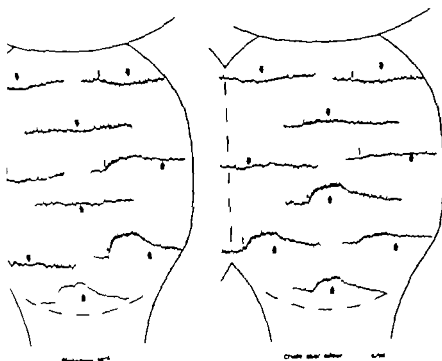
In this presentation the choice of stimuli has been limited to a) the following L-α-amino acids: ... glutamine, histidine, lysine, methionine, serine, tryptophane and tyrosine in the concentration 10^{-4} M and b) crude odorant samples from the same populations as the experimental animals. The "fish odour" samples were chosen in order to look for possible species- and population-specific d... in the responses as they were suspected to contain population specific pheromones (see Dö... The crude fish odour samples were tapped from the basins where the fishes were kept ... presented in half decade concentration steps from undiluted to below threshold concentrations. ... centrations of the "fish odours" were estimated in terms of the number of fishes i... each basin water flow through it (fish/ml). The concentrations used ranged from 3.6 to 0.02 fish/ml. ... "crude fish odour" were tested in all expts.

Experimental procedure

Responses have been recorded from both olfactory bulbs stimulating the ipsilateral olfactory ep... The recording electrodes were placed in 15 positions covering the dorsal surface of the bulb. Th... side and the interior of the bulb were reached by penetration. Responses from both medial ... positions were recorded in each experiment. In all the chars the responses were recorded from 2 ... simultaneously, one electrode was kept stationary serving as a control of stimulation and of the ... of the preparation.

The blood flow in the brain surface vessels was regularly inspected as control on the circulation, especially during the manipulation of the electrodes.

Each kind of stimulus was presented in the order of ascending concentration, each concentration



Typical DC recordings of the responses in selected positions on the dorsal side of the lef olfactory ' of char during odour stimulation of the ipsilateral olfactory epithelium ith) glutamine, 10^{-4} M,) crude char odour" 0 6 fish s/ml The recordings are displayed at their respective recording positions the outline sketch of the left olfactory bulb The solid arrows pointing at each recording are placed (actual recording area. The idth of the arrow shafts is 1 The short vertical bars marking the onset , the stimulation period are 100 μV negative upwards.

se the differences in polarity and magnitude of the DC potential shift as well as those of the induced

ral surface and in the center of the bulb was related to those recorded from the dorsal ace. These recordings were also interesting in view of the current ideas about the site of gin of the responses (see Discussion). Fig. 3 displays results from such an experiment. electrode was pushed from the center of the dorsal surface of the olfactory bulb, vertily through the bulb until touch with the braincase bottom and withdrawn in 0.1 mm steps. pression of the tissue during the penetration made the determination of the position the electrode tip uncertain. The recordings, therefore, were only made during the withwal of the electrode.

The figure confirms the odour specific differentiation of the bulbar responses to glutamine crude char odour" It shows that the response to glutamine is far stronger at the ventral than central on the dorsal and in the center of the bulb while crude char odour" tends give more uniform response distribution. In the center of the bulb all induced waves owed positive sharp peaks while the DC shift almost disappeared. The changes of polarity e sharp peaks occurred approximately 0.3 mm from the ventral and dorsal surfaces

forward on the olfactory nerve. They were therefore not projections of the electroolfactogram. Th nomenon was regarded as an artifact, but being contemporary with the true odour stimulation with the measurements of the DC response and may in some recordi gs have produced a ... +100 μV

The AC component of the recordings changed at stimulation from irregular spont activity amplitude range 20-90 μV peak to peak, into regular induced waves with a peak amplitude of up to 600 μV and frequency range of 3-6 Hz. The induced waves had an asymmetrical waveform with sharp and blunt peaks. Spindle-like bursts ... tions were rare In some cases the induced waves showed alternating high and low ... tudes. These more sporadic phenomena have not been subject to analysis on the work

Variability in response

Stimulation with a given odorant at a certain concentration induced responses which different recordings in different regions of the bulb. Other odorants elicited other p... of response distribution. The most prominent differences in response distribution found between any of the "crude fish odours" and any amino acid. The variations ... features of the responses are illustrated in different ways in Fig. 2-7 Except for Fig ... of the figures displays results from one preparation only

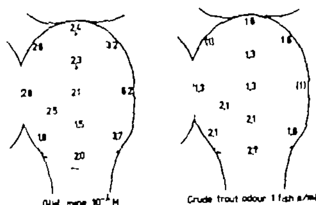
Fig. 2. shows the qualitative differences in the surface responses to stimulation ... amino acid (glutamine) and a "crude fish (char) odour" at selected recording ... covering the dorsal surface of the olfactory bulb The differences refer to the magnitude polarity of the DC-shifts, the relative amplitude of the induced waves and the polar... the sharp peaks. The figure is composed of recordings from 4 consecutive char prepar... with qualitatively and quantitatively similar responses in common control positions.

Glutamine gave a pronounced negative DC shift in the rostrolateral half and a ... positive shift in the caudomedial half The induced waves had largest amplitudes ... showing negative sharp peaks. They were nearly indetectable along the dorsal midline weak with positive sharp peaks at the medial side. Thus the polarity of the sharp p... followed that of the DC potential shift except in the caudolateral corner of the bulb ... negative sharp peaks were superimposed on a positive DC shift In this corner and ... extreme rostral end of the bulb the spatial difference in distribution of the AC-response the DC-response was clearly seen

The distribution of responses to crude char odour" was different The DC shift as ... the induced waves were largest in the rostral parts of the bulb. In the same region both DC shift and the sharp peaks of the induced waves were negative. Also with this ... a slight separation of the two response components, the DC shift and the induced w... were seen. The largest induced waves were found a little more caudally than the greatest potential shift

The recordings which were all made with two electrodes simultaneously showed that sharp peaks of the induced waves always appeared exactly in phase or in counterp... throughout the entire olfactory bulb.

Penetration experiments were performed to see how the distribution of responses ...



...stribution of the responses to glutamine, 10^{-4} M and "crude trout odour" 1 fish s/ml, on the left ... bulb of ... trout. The numbers give the amplitudes of the induced ... ves relative to that of the ...ous activity. The ... and − signs indicate the polarity of the sharp peaks. No sign indicates sym... oscillations. () indicates that induced waves were not detected.

...ntrations used, 10^{-4} M the curves being approximately linear. The slopes were steepest ...e corresponding responses at higher concentrations were greatest at the lateral posi... the DC shift and the sharp peaks of the induced waves both being negative. At the ...l position the responses were weaker and of the opposite polarity. At the two recording ...ons along the dorsal midline of the bulb the responses were small and varied little with ...ntration. Similar results were also found for the other amino acids used.

...e results from stimulation with "char odour" are less conclusive as the concentration ...was only 1½ decade. Only at the rostral recording position the response magnitude was ...y related to stimulus concentration. At this position the results parallel those found for ...tamine at the lateral position. At the medial position the induced waves were weak and ...ed no obvious asymmetry.

...together the curves indicate that provided the stimulus and the recording position ...in unchanged, the magnitude of the DC shift is approximately proportional to the ...itude of the induced waves above the spontaneous activity.

...s indicated in Fig. 5 there was an approximately linear relationship between the magnitude ...e DC shift and the amplitude of the induced waves. This relationship is also illustrated ...g. 6. In addition this figure shows in a quantified manner an example of the spatial ...rence in the magnitudes of the two response components also demonstrated in Fig. 2 a. ...ecordings from homologous positions in the ipsi- and the contra-lateral bulb during ...lateral stimulation of the olfactory epithelium showed that the spatial distribution of ...ponses to any given stimulus were mirror images of each other and that the responses ...reased with increasing stimulus concentration in a similar manner in homologous posi...ns. In reliance on this symmetry results from the right bulb have been presented in this ...icle as if they were all obtained from the left. This is the case in Fig. 3 and 5 and for some ...recordings in Fig. 2.

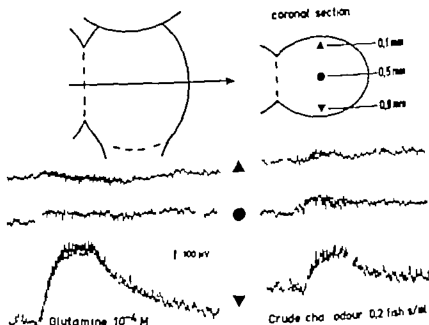


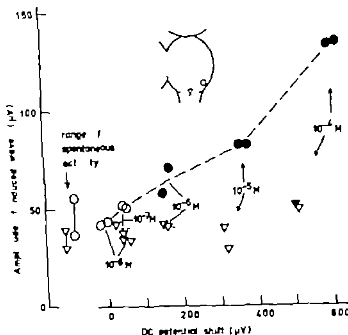
Fig. 3 Recordings from penetration ertically through the center of the olfactory bulb of a char T sketch on the top shows the appro imate position of the electrode tip for 3 selected couples of record Note the lack of significant DC sh fts in the center and the changes i the polarity of the sharp peak

using crude char odour" just ventral to the center of the bulb when using glutamine stimulus. No histological investigation has been made in this study The different record positions can therefore not be exactly related to any specific layer of the olfactory bulb. T penetrations caused some damage to the bulb. In spite of the greater responses on the ve side most of the recordings were therefore made from the dorsal surface of the bulbs.

Measurements of the response amplitude have been made on a number of fish. Fi displays the results from AC recordings on a trout The spatial variation in the indu waves during stimulation with glutamine and "crude trout odour" in a single preparation seen The responses have been quantified as the ratio of the peak to peak amplitude of t induced waves to that of the preceding spontaneous activity The polarity of the sharp pe is shown The results are plotted on the dorsal surface of the left olfactory bulb. For b stimuli the highest relative amplitudes are found together with negative sharp peaks. T lowest amplitudes correspond roughly to the zone of undeterminable polarity As to t odour specific differences in the response distributions, Fig. 4 shows essentially the sa picture as Fig. 2.

The relationship between stimulus concentration and response magnitude at dif recording positions is demonstrated in Fig. 5 The figure shows the stimulus/response cu obtained from 4 positions on the olfactory bulb during stimulation with different concen tions of methionine and "crude char odour" Each point represents the mean value of t results from 2–6 recordings.

The responses to methionine show the clearest picture. The stimulus/response cu obtained at different positions were not parallel but radiating from a common origi 10⁻ M The slopes of the curves above this point correspond to the responses at the hi



Comparison between the amplitude of the induced aves and that of the DC potential shift in ... recordings from two portions on the left olfactory bulb of clear during stimulation ... in 10^{-8}–10^{-4} M concentrations. Symbol shapes refer to the recording positions on the sketch. ...mbols indicate that induced waves ere clearly seen

...ce of mixture of amino acids produced spatially different response patterns, mixtures ...al parts of the same amino acid solutions in the concentrations given in the figure ...ested. The response to the mixture of all the 8 amino acid solutions is shown at the ...n of Fig. 7. The mixtures elicited much larger responses (measured as DC-shift) than ...ost potent of their constituents, still without any sign of spatial shift. However the ...se to any of the mixtures was less than that expected if it had been a sum of independ... ...sponses to each of the amino acids at their actual concentrations in the mixtures.

... "fish odour" samples usually showed small but clear differences in their spatial ...nse patterns when tested on the same animal. In the whole material, however the ...ences were overshadowed by the individual variation of the preparations. Different ...r samples from the same population and therefore presumably the same "fish odour" ... gave different response distributions as well. The response distribution to given ... odour" could therefore only be coarsely predicted from the responses to other "fish ...r samples and the amino acids. Nor could the response distribution give any safe ...ation of which population an unknown odour sample came from.

...idual ...ariations

... results often differed from one preparation to another as if the bulbs were slightly ...ted or rotated in relation to each other. This was most clearly seen when individual

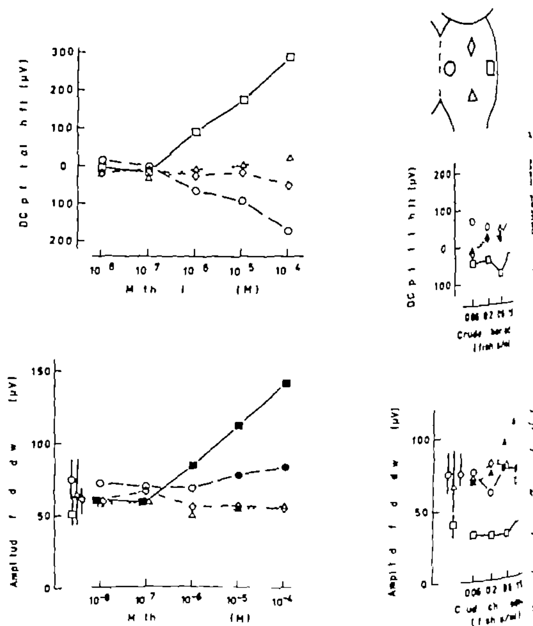


Fig 5. The magnitude of the DC potential shift (upper two diagrams) and the amplitude of the AC component of the record ng (lower diagrams) i 4 posit ons the olfactory bulb of a char during stimuli with methionine (right) crude char odour" (left) t different concentrations. Graph symbols correspond to the recording positions shown on the sketch in the upper right corner
In each of the two lower diagrams the mean nd range f the spontaneous act vity t each position shown to the left Filled symbols indicate that induced waves were clea ly seen. The polarity of the peaks is shown at the right end of the curves, ± indicating undeterminable polarity

Similarity between stimuli

Only slight differences were seen between the responses to the eight amino acids used in the present study No differences in their spatial response distributions were seen. The differences, as demonstrated in Fig 7 were limited to small variations in the AC to DC amplitude ratio.

potential spread in a volume conductor a weaker positive potential appeared on the se side of the bulb in relation to the negative zone. A prominent positive DC shift was spected in the center of the bulb. This was, however not found. In the present penetra- speriments no DC shift was evident in the center It is likely therefore, that a part of C signals reflects events in structures confined to the surface of the bulb, e.g. the ol- y nerve terminals. The question remains if the slow positive DC potential shift ob- l caudally on the bulb reflects the contribution by postsynaptic elements like the l cell dendrites or has an origin entirely different from the rostral negative one.

uced waves may be of relatively constant amplitude throughout the stimulation, ating amplitude, or appear as spindle-like bursts of waves (Ottoson 1959 a). The ating frequency is species- and temperature-dependent (Dupé and Godet 1969 Gray- , Low Rogers and McLennan 1970, Døving and Belghaug 1977) ranging from about z in mammals (Adrian 1950) down to 3 Hz in fish bulbs at a temperature of about 5°C. a Baumgarten, Green and Mancia (1962) have made simultaneous surface and depth dings from the olfactory bulb of the rabbit. From the surface all through the external form layer down to the mitral cell layer the induced waves were exactly in phase but creasing amplitude. In the mitral cell layer the waves almost vanished, and in the internal form layer the induced waves showed the opposite phase as the surface waves did. The ced waves thus did not behave like a projection of moving dipoles, but like a sum of onary oscillating ones. Unit activity was phase locked in such a way that action po- ials in the cells of the different layers occurred predominantly at the negative going phase he induced waves recorded from the same layer Their results indicated that the induced es ere due to synaptic events between secondary and tertiary neurons.

n the present study the occurrence of negative sharp peaks in the induced waves is closely ciated with the area giving the highest amplitude in response to the same stimulus. The ultaneous recordings referred to in the present work clearly show that induced waves h positive sharp peaks are just the same signals seen from the reverse, the greater distance m the ave generating layer being responsible for the lower amplitude. The different ble of the induced waves from that of the DC component shows itself in the present results the difference in their spatial distribution.

cific spatial distribution of responses

ry olfactory stimulus influences the olfactory bulb in such a way that a synchronized os- lating activity is produced in a specific population of secondary neurons. The specific lection of activated neurons tells the fish about the odorant to which it is exposed. From e experimentor s point of view the surface potential changes reflect the localization of the naptic events connected to the specific activity

In most preparations the results indicate that the bulbar information treatment on amino cids takes place mainly in the lateral and perhaps the ventral parts.

From single cell recordings in the olfactory bulbs of goldfish (Meredith 1974) and rainbow out (MacLeod 1976) it has been found that the amino acids have unequal, yet overlapping euronal representation in the bulb. On the primary level, cross-adaptation studies on the lfactory epithelium of Atlantic salmon (Sutterlin and Sutterlin 1971)

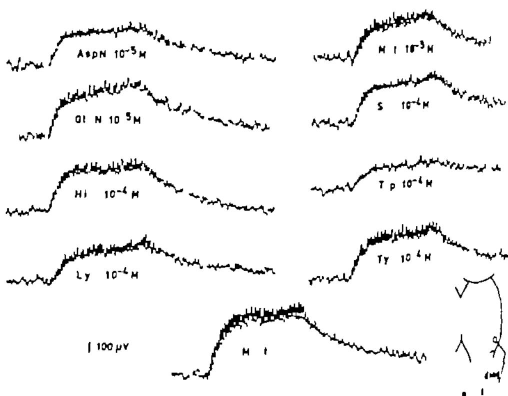


Fig. 7 Comparison of the responses t 8 amino acids with the one t the mi ture of even parts of th solutions. Note the differences in the AC to DC amplitude ratio for the different amino acids greater response magnitude of the mixture.

division lines between regions of positive and negative sharp peaks of the induced were estimated The sensitivity of the preparations varied from one individual to In general the most sensitive preparations were found during the summer and the sensitive during the early spring. Minor stimulus specific differences in the sensitivity also seen. No conclusion on this observation can be drawn however as the exper were performed too irregularly which may have caused differences in the experiment nique

Discussion

On the origin of the bulbar potentials

Olfactory bulb responses in fishes show the same general characteristics as those higher vertebrates. The surface potential changes induced in the olfactory bulb by olfactory stimulation consist of two separable events (Ottoson 1959 b), a sustained or DC potential and the "induced waves" (Adrian 1950).

Ottoson (1959 b) demonstrated that centrifugal stimulation of olfactory tract blocked the induced waves leaving the DC component of the olfactory response intact. concluded that the results spoke in favour of a presynaptic origin of the slow response, did not exclude the contribution by postsynaptic elements. This view is supported by present results. The greatest DC shifts found were constantly negative. As expected accord

otential spread in a volume conductor a weaker positive potential appeared on the side of the bulb in relation to the negative zone. A prominent positive DC shift was ected in the center of the bulb. This was, however not found. In the present penetra- eriments no DC shift was evident in the center. It is likely therefore, that a part of signals reflects events in structures confined to the surface of the bulb, e.g. the ol- nerve terminals. The question remains if the slow positive DC potential shift ob- ccasionally on the bulb reflects the contribution by postsynaptic elements like the cell dendrites or has an origin entirely different from the rostral negative one.

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between receptors sensitive to some of the amino acids. In the present study the re[...] to the different amino acids show slight differences in the AC/DC amplitude ratio [...] they show the same response distribution. The tests with the amino acid mixtures [...] response magnitudes intermediate between that expected if all the amino acids were [...] as one and that if they had no receptor site in common These observations are co[...] with both the difference and the similarity in the neuronal representation of these odo[...]

The rostromedial third of the bulb is presumably the main site in the bulbar level [...] formation treatment concerning the 'fish odours The area is overlapping with the [...] rostral part of the amino acid field Skin mucus samples of salmonid fishes contain [...] free amino acids (R Selset 1975 personal communication) as well as components sup[...] to act as pheromones (Nordeng 1971 Døving, Nordeng and Oakley 1974). As an ol[...] stimulant the solution may thus be multipotent, causing the response distribution to [...] as intermediary between that of the amino acids and that of the pure "fish odour" [...] stimulus components causing medially located response patterns are not yet identifi[...] odorant samples tested, elicited responses which were too much alike to make the prep[...] suitable as a bioassay for population specific odorants It is even not evident that [...] components are unique to the fishes. The term "crude fish odour" has, however be[...] in this article due to the origin of the actual odorant samples used and in the absence [...] precise terms.

As to the hypothetical pheromone function of such components it is interesting [...] the response distribution they cause This distribution corresponds well with the [...] the olfactory bulb which according to the anatomical investigations (Sheldon 1912, H[...] 1920) has a direct connection with the magnocellular part of the preoptic nucleus in the [...] pothalamus. The electrophysiological work by Kandel (1964) indicates that through [...] nection pituitary activity may be influenced by olfactory stimulation. The olfactory [...] most likely to produce endocrine changes in the fish should consequently be a "fish [...]

Spatial variation in the response distributions with different stimulus concentrat[...] not been clearly observed, but is likely to occur with crude mixtures like the "fish o[...] components giving different spatial response patterns reach their threshold or sa[...] concentrations at different mixture concentrations. This may be the explanation [...] indistinct results to stimulation with "crude char odour" in Fig. 5 Such effects m[...] occur using pure substances like amino acids, as each of these may act as an odour [...] due to different receptor specificity in different parts of the molecule.

The magnitude of the bulb surface response has been used by several authors in ev[...] of olfactory sensitivity to certain odorants in different individuals of salmonid fis[...] stimulus/response curves show that this evaluation may be fairly reliable as long as c[...] recording positions and stimuli are used. The spatial variation in the slope of th[...] implies, however that the sensitivity of the olfactory organ in terms of threshold [...] concentration may be the same in different individuals even if response magnitude [...] threshold concentration is highly different, particularly near to the dorsal midpo[...] bulb which is the most accessible part of it Individual variations in the orientatio[...] bulb with regard to the odour specific areas or small variations in the positionin[...] recording electrode may therefore, produce marked changes in t[...] ampl[...]

…present results confirm that the principle of spatial differentiation of odour specificity …l for teleosts as well as for air breathing vertebrates. On the basis of the distribution … ojection of the primary olfactory neurons in the smelt (*Osmerus eperlanus*), Holmgren … ted in 1920 that an olfactory map, "eine Riechkarte" existed on the olfactory bulb … regard to the fish he was half a century ahead of his time.

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…ant to thank K. B. Døving, P. S. Enger and J. Jansen, Jr, for their valuable advice and suggestions … the preparation of this study

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Neuroeffector maturity of portal veins from newborn rats, rabbits, cats and guinea pigs

By

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Abstract

STAGE (MCMURPHY), D and B LJUNG. *Neuroeffector maturity of portal veins from rats rabbits cats and guinea pigs* Acta physiol scand 1978 *102* 218-223

Isolated portal veins from newborn and adult rats, rabbits, cats and guinea pigs were studied in vitro. Spontaneous activity and induced responses to transmural nerve stimulation (NS), noradrenaline, acetylcholine and $BaCl_2$ were quantitated In agreement with our previous observations the portal vein of the newborn rat was found to lack spontaneous activity and responsiveness to applied stimuli. The newborn rabbit portal vein showed inconsistent and weak responses. In the neonatal cat and guinea pig portal veins the pattern of the spontaneous activity and induced responses resembled those found in veins from adult animals. It is concluded that the portal vein maturity at birth in several respects correlates with the level of somatic development of the animal. Furthermore, the previously described time course of neuroeffector development of the portal vein during the postnatal period in the rat corresponds to prenatal events in cats and guinea pigs which are more highly developed at birth Consequently the lack of responsiveness of the portal vein during the first week of life does not seem to be secondary to the hormonal or hemodynamic adjustments during the neonatal period. Rather the postnatal development of the portal vein occurs according to a genetically predetermined schedule, possibly in parallel with the ontogeny of vascular control in general

Spontaneous phasic activity is a characteristic functional feature of the longitudinal muscle layer of the adult rat portal vein In a previous study (Ljung and Stage 19 found that coordinated myogenic contractions did not appear in the developing vein until the beginning of the third postnatal week. Weak responses to exogenous noradrenaline (NA) and to transmural nerve stimulation (NS) could first be elicited middle and the end of the first week of life, respectively The sensitivity to these stimulation increased coincidentally to the onset of spontaneous activity during postnatal week The most likely explanation for the delayed onset of responsiveness observed changes in sensitivity to adrenergic stimulation was considered to be the normal maturation process of smooth muscle and nerve development. However possible, but less likely explanations, such as hormonal influences during the lactat and secondary hemodynamic changes at birth in the splanchnic circulation, mu sidered as well.

...e present expts. the neuroeffector function of portal veins from newborn and adult ...bbits, cats and guinea pigs have been compared. The offspring of these animals ...t varying degrees of maturity at birth but with presumably similar exposure to ...nal influences and comparable hemodynamic alterations at birth.

Methods

...l portal vein preparations from newborn (0–7 days of [illegible]ge) and adult animals of 4 different species ...rable maturity at birth [illegible]ere studied. Division of the animals by age and mean [illegible] eight [illegible] as as follows. ...rague-Dawley)—6 newborns (10 g), 4 adults (250 g); rabbits—7 newborns (50 g), 3 adults (1.5 kg); ...newborns (120 g), 1 mother cat (4.2 kg); and guinea pigs—6 newborns (95 g), 2 mothers (760 g). ...imals were killed by decapitation, [illegible] blow to the head, or an overdose of ether. The portal vein [illegible] as ...d out and cut open longitudinally. I[illegible] most cases the entire vein was used, but in adult guinea pigs, ...and cats longitudinal preparations measuring 10–15 mm in length and 2–3 mm in width were used. ...ue [illegible] as mounted under tension in an organ bath containing 40 ml of Krebs solution (composition ...ow). Isometric longitudinal contractile activity [illegible]as recorded on [illegible] Grass polygraph (model 7) by ...of [illegible] Grass force-displacement transducer (FT 03C). The level of passive tension [illegible] as determined ...ually for each preparation by adjustment of its length to that level at [illegible] hich optimal active force was ...ted by 10 [illegible] periods of transmural field stimulation [illegible]t an impulse rate of 8 Hz. T[illegible] non-responding ...veins from newborn rats and rabbits [illegible] passive force of 1 and 2 mN, respectively [illegible] was applied. An ...modation period of 60 min was allowed before beginning the experiment.

...l expts. [illegible] are performed according to the following protocol. At the end of the accommodation ...phasic spontaneous activity was quantitated. Then the adrenergic nerve plexus of the portal vein ...which was activated by transmural field stimulation (NS) during [illegible] 1 min period by use of [illegible] Grass ...ator model S4B, [illegible] hich provided square wave impulses of 0.8 ms duration and 15V in amplitude, ...in platinum electrodes mounted on either side of the preparation, by means of [illegible] constant voltage ...t. The impulse rate of 32 Hz [illegible] as chosen to ensure [illegible] maximum or supramaximum response to trans... field stimulation (cf. Leung et al. 1975). Responses were then elicited by 3 min exposure periods to ...renaline (NA) 10 µM, tyramine (Tyr) 0.1 mM, acetylcholine (ACh) 1 mM and $BaCl_2$ 5 mM. These ...ntrations were chosen from preliminary expts. to provide maximum contractile responses t[illegible] each ...of stimulation. Adequate time was allowed between drug exposures for complete return to baseline ...ty. At the end of the experiments the length of the mounted tissues [illegible] as measured by means of a ...tion microscope with [illegible] micrometer eye piece. The preparations were then removed, blotted between ...papers and [illegible] eighed on [illegible] Cahn microbalance. The values for weight and length [illegible] ere used to calculate ...tissue cross-sectional area, assuming [illegible] density of 1.

...ak force values for all responses were calculated in absolute terms as maximum force (mN), maximum ...ss (mN·cm⁻²) and also expressed as [illegible] percentage of maximum NA response. Comparison of the mean ...s between the two age groups [illegible] as made by use of Student [illegible] t-test in groups [illegible] ith adequate numbers ...any such [illegible] comparison. A statistically significant difference was considered to exist between the com...d groups [illegible] hen the p value was less than 0.05.

...e standard Krebs solution had the following composition in mM: NaCl 122, KCl 4.73, $NaHCO_3$ 15.5, ...PO_4 1.19, $MgCl_2$ 1.19, $CaCl_2$ 2.49, glucose 11.5 and $CaNa_2$-versenate 0.026. I[illegible] was modified for use ...veins by the addition of 30 mM sucrose. The bath solution [illegible] as bubbled with 4 per cent CO_2 in O_2 to ...obtain [illegible] pH of 7.35 at the temperature of 38 C. Drugs used were l-noradrenaline HCl (l-arterenol, ...m Chem. Co., St. Louis, Mo., U.S.A.), tyramine chloride, acetylcholine chloride and $BaCl_2$ (E. Merck, ...mstadt, Ger.). Freshly prepared, saline-diluted drugs in [illegible] volume of 0.4 ml were injected directly into ...40 ml bath to give the desired concentration.

Results

Maturity at birth. The great variability in the maturity level of the four different animal ...cies at birth was readily [illegible]pparent by examination of their physical appearance. At the ...st immature level on the developmental scale was the furless, sightless and immobile

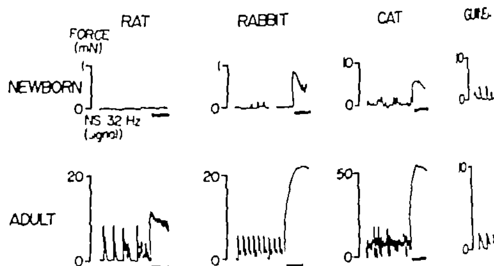


Fig. 1 Experiment I recordings of spontaneous activity and responses to transmural field stim supramaximum impulse rate (32 Hz). Note lack of responsiveness in neonatal rat and poor neonatal rabbit portal vei

newborn rat which is born after a gestational period of 21 days and is totally dep the mother for survival At the other end of the scale was the fur covered, open mobile newborn guinea pig, which is born following a gestational period of 68 furless and sightless newborn rabbit with a gestational period of 31 days and the fur covered newborn cat with a gestational period of 63 days fit in between the guinea pig on this developmental scale Thus, placement of the four species in ascending maturity at birth is as follows rat, rabbit, cat and guinea pig.

Spontaneous activity and responses to nerve stimulation The experimental rec Fig 1 illustrate species variation in onset, pattern and magnitude of spontaneo and response to nerve stimulation at a frequency of 32 Hz in newborn as comp adult portal vein preparations. Complete lack of activity is noted in the port the newborn rat The portal vein of the newborn rabbit sometimes exhibited of spontaneous phasic activity occurring irregularily at the beginning of the e and in most cases a clear cut but weak response to nerve stimulation at 3 obtained All preparations from the newborn cats and guinea pigs manife spontaneous phasic activity and neurogenic responses. Although the pattern contractions varied, forceful spontaneous contractile activity appeared throu expts. Likewise the magnitude of response to maximal NS was greater than in th rat and rabbit.

All adult preparations exhibited spontaneous activity and NS response. As see the frequency and magnitude of spontaneous contractions were greater in the a vein than in the vessels from newborn animals. It is not possible to quantitativel contraction amplitudes or degree of synchronization by the tracings of Fig. 1 tudinal strips of adult portal veins from the rabbit cat and gu were used

circumference of the vessel was included in all preparations from newborn animals : adult rat.

se NA. The relationships between the number of tissues responding to exo- (10 μM) and the total number studied and the peak force response magnitude Table I. In 4 out of 6 portal veins from rats studied during the first week of life 7 rabbit portal veins no NA responses could be elicited. In all other preparations one from newborn kittens and guinea pigs clearcut responses were obtained.

mplitude of spontaneous activity and responses to NS tyramine acetylcholine and ble 1 the number of portal vein preparations which exhibited spontaneous responded to transmural nerve stimulation (32 Hz), to tyramine (0 1 mM) to e (1 mM) and to $BaCl_2$ (5 mM), respectively ha e been expressed as a fraction number of tissues studied. In some experiments the whole sequence of non-stimulations was not applied, hence the variation in number of observations. In where a NA response was obtained the peak force of spontaneous activity and the onses amplitudes were expressed as a percentage of the maximum NA response. significant differences between such relative response values of portal veins from newborn animals were found for spontaneous activity nerve stimulation and the rat and for nerve stimulation in the rabbit. It is of particular interest that the responses of newborn cats and guinea pigs corresponded to those of adult ts.

Discussion

ortal vein did not respond to nerve stimulation during the first week of life and to exogenous NA were only obtained in those rat preparations which were studied e end of the first week of life. These findings are in accordance with our previous ing and Sorge 1975) where the ontogenetic development was examined in several vein preparations from each day during the first 6 postnatal weeks. It was found ge at onset of responses to NA, to ACh and to nerve stimulation was 4 4 and 6 days, ly Furthermore, phasic spontaneous activity occurred rather abruptly at the 16th day This functional development is paralleled by morphological differentiation the and in-growth of terminal adrenergic nerve fibers (Lundberg *et al.* 1976). It ar that the neuroeffector maturation of the portal vein occurs postnatally in the rat. animals, notably the guinea pig and the cat, spontaneous myogenic activity of the in and responsiveness to external stimuli developed earlier than in the rat. It ap- fficient to study a limited number of portal ein preparations from these newborn ones we found that they do exhibit spontaneous activity and do respond to ad- stered during the first few days of life. Moreover we only included few adult since their portal veins have previously been studied in these respects (for ref see ng 1970). The rather limited number of observations precludes any detailed quantita- mparisons but it seems possible to conclude that in the cat and the guinea pig the fector function of the portal vein is completely developed in all respects at birth since the relative magnitude of spontaneous activity and responsiveness to variety latory stimuli acting at different levels in the neuroeffector chain are comparable

TABLE I (A) Responses to exogenous noradrenaline of portal veins from various newborn and adult animals expressed as relative number of tissues responding to the agonist and peak force (mN) and peak tension (mN/mm^2) of responding preparations. Mean ± S E. Note the portion of the media circumference was included in the preparations of portal vein from rabbits, cats and guinea pigs. Statistically significant differences ($p < 0.05$) between

	Rat				Rabbit		
	Newborn		Adult		Newborn		A[illegible]
A							
Noradrenaline 10 μM							
No responding/No tested	./6		4/4		5/7		[illegible]
Max force (mN)	0.11±0.01		10±0.9		0 54±0 3		[illegible]
Max tension (mN/mm^2)	3.3±3		2.±3		1±1		[illegible]
B							
No responding/No tested max force (max NA response)							
Spontaneous activity	0/6	0	4/4	55±6	4/7	18±8	[illegible]
Nerve stimulation (32 Hz)	0/6	0*	4/4	96±2	5/7	84±6	[illegible]
Tyramine 0.1 mM	0,6	0	4/4	75±9	1/5	17	[illegible]
Acetylcholine 1 mM	3/6	114±14	2/2	105±5	3/5	90±5	[illegible]
$BaCl_2$ 5 mM	3/6	10.±2	4/4	109±3	3/5	85±5	[illegible]

(Table I) (Gerova *et al* 1974). Naturally maturation of the smooth muscle may that increased tension responses, expressed as force per smooth muscle cross sectio are obtained with advancing age (see Ljung and Stage 1975). Indeed, the tensio (mN/mm^2) given in Table I indicate that increase in contractility does occur during development in all species studied except the guinea pig. However the cross secti was calculated from values for weight and length in our experiments and thus the di of the actual effector tissue, *i.e* the smooth muscle of the longitudinal media layer been determined directly The relative amount of longitudinal smooth muscle in the c wall of the portal vein can be expected to vary during development (*cf* Lundberg *et* Consequently a definite conclusion regarding changes in vascular smooth mu tractility cannot be drawn from the present results alone.

In the newborn rabbit portal vein weak spontaneous activity was found in 4 preparations. Furthermore, adrenergic responses were present in the majority of the greater responsiveness seems to occur in the newborn rabbit portal vein than in the rat

Altogether the present results strongly indicate that the degree of portal vein m birth is related to length of gestational period and stage of development of the fo studied Such variation in vascular development with gestational length is in agreem a recent *in vitro* study on the carotid artery of the fetal lamb (Su *et al* 1977) where ence of functioning neuroeffector control was demonstrated already at the beginni second trimester Consequently the dramatic changes which occur in the portal ve rat during the first few postnatal weeks seem to take place during fetal develop animals which are more highly developed at birth such as cat, guinea pig and sheep the rabbit portal vein assumes an intermediate position This seems to rule out stages of postnatal development previously observed in the rat portal vein are seco

adult tissues are indicated by asterisk (*). † indicates that significance test has not been performed. (B) Spontaneous activity and induced response to various stimuli expressed as relative number of tissues responding and as a percentage of the maximum response to exogenous noradrenaline. Mean ± S E.

			Guinea pig	
	Adult		Newborn	Adult
	1/1		6/6	2/2
	48		6 7 ± 1	10 ± 1
	52		12 4 ± 2.4	14 8 ± 3 6
†	1/1	38	6/6 36 ± 8	2/2 25 ± 1
†	1/1	117	6/6 103 ± 5	2/2, 86 ± 28
4 †	1/1	73	4/4 52 ± 9	2/2, 30 ± 12
3 †	1/1	88	6/6 74 ± 7	2/2, 55 ± 3
13 †	1/1	92	6/6, 59 ± 7	2/2 59 ± 6

…nal influences during lactation or hemodynamic alterations at birth but reflect true …netic development. Thus, it appears that neuroeffector function of the rat portal …develops in accordance to a genetically predetermined schedule, possibly in parallel …he advent of efficient myogenic and neurogenic control of the vascular system.

…e grateful to Miss Ann Kjellstedt for technical assistance and to Miss Lena Zadén for secretarial aid. …ted by Wilhelm och Martina Lundgrens Vetenskapsfond, by the Swedish Medical Research Council …and by AB Hässle.

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An electronic differential pressure flowmeter and a resistance for continuous measurement of vascular resistance

By

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Abstract

GRÄNDE, P.-O. and P. BORGSTRÖM. *An electronic differential pressure flowm resistance meter for continuous measurement of vascular resistance.* Acta phys 1978. *102*. 224-230.

An electronic differential pressure flowmeter for mean blood flow measuring and its use meter for continuous recording of vascular resistance is described. The flowmeter allows linear registration also in the small flow range (< 10 ml/min), thus appropriate for use in mental animals.

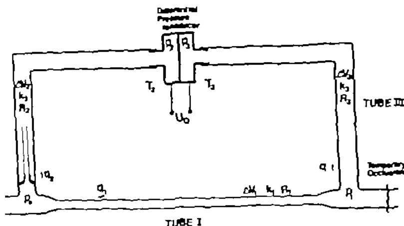
Key words. Blood flowmeter, vascular resistance meter, differential pressure flowmeter

One of the most important variables in the peripheral circulation is vascular r It is usually determined by manual calculation of the ratio of mean perfusion and mean blood flow. This is, however, a time-consuming process which for reasons only gives scattered information. Continuous recordings of vascular could be readily obtained by feeding the output signals from a differential press ducer and a flowmeter into an electronic divider circuit. Instuments with high acc stability must be used, however, to obtain reliable continuous measurements of resistance.

A problem in this context is encountered in studies on small experimental ani minute tissue blood flows in the range below 10-15 ml/min, since most con flowmeters, like the electromagnetic one, show poor technical characteristics in range. This paper describes an extracorporeal differential pressure flowmeter for ment of minute blood flows and its use for continuous recordings of total and s resistances in the vascular bed of skeletal muscle in the cat.

The differential pressure flowmeter

Theoretical considerations. The flowmeter device consists of an extracorpore branching rigid tube with uniform cross-sectional area across   ferential p



[Sch]ematic illustration of the differential pressure flowmeter under temporary occlusion of flow ...rter currents of tubes I, II and III, here ΔV = induced volume change, R = tube resistance, ...n, T time constant and k tube elasticity moduli. Note the special construction to obtain ...ched by making the relationship $R_2 = R + R_0$ and $k_2 = k_3$ (see text).

...usly recorded. A mathematical analysis of the pressure–pulsatile flow relationship ...ewtonian fluid in such a system was made by Fry Mallos and Casper (1956) by ...nation of Navier Stoke s equations

$$p(t) = \varrho\ \Delta x\ du/dt \quad a\ u(t),$$

...(t) is the pressure difference, as function of time, over the distance (Δx) between ... measuring sites, u(t) blood elocity as a function of time, ϱ fluid density and a a ...t relating the frictional pressure drop. The velocity profile is assumed flat. When ...ing mean flow in the present system, the term $\varrho\ \Delta x\ du/dt$ can be ignored since it is ...ingly small compared to a u(t), (*cf* McDonald, 1974). Thus

$$p(t) \quad a\ u(t),$$

...th constant tube cross-sectional area

$$p(t) \quad b\ q(t)$$

... b is a constant and q(t) volume flow as a function of time. This equation is approxi...y valid also for a non-Newtonian fluid, like blood for the tube dimensions and flow ...ities used in the present device (see below).

...e basic principle of the flowmeter is shown schematically in Fig. 1 It consists of a ... tube (I) diverting blood flow (q_1) from the proximal to the distal end of a divided ...y The pressure drop over tube I is measured with a differential pressure transducer ...irtually no membrane displacement (National Semiconductor Corp., LX1601D) ...ubes II and III, the former supplied with an inner tubing of smaller size for a special ...ose (see below). It is essential that the pulsatile pressure at the entrance of tube II is ...mitted to either side of the differential pressure transducer without significant phase ... This implies that the time constant (T_2) of tube II should be identical to that (T_3) of

tubes I + III which can be accomplished by choosing appropriate values for the three resistances, R R_2 and R and for their elasticity moduli, k_1 k_2 and k_3 (see Fig. 1) acc to the calculation below Since the time constants are independent of flow the calc refers to the zero mean flow situation accomplished by temporary occlusion of the distal artery (Fig. 1) Pulse pressure will then cause minute flows, q q and q_3 the three limbs of the flowmeter p_0, p p_2 and p_3 denote pressures at the sites in Fig. 1 ΔV ΔV and ΔV the pressure induced volume changes of tubes I, II and k_1 k_2 and k their elasticity moduli Then $dp = k_1 dV$ $dp_2 = k\, dV_2$, dp_3 $k_3 dV$ $dV_1/dt = q_1 - q$ $dV_2/dt = q$ $dV_3/dt = q_3$.

The relation between flow resistance, and pressure drop for the three tubes expressed

$$q\ R\ = p_0 - p \quad q\ R_2 = p_0 - p_2 \quad q_3 R_3 = p_1 - p_3$$

If $1/k\ \approx 0$ (*i.e.* tube I is made rigid), the equations above give, after Laplace transformation, the following solution

$$p = p\ \frac{1}{1 + \frac{R_2}{k} s} = p_0 \frac{1}{1 + T\ s}$$

$$p = p_0 \frac{1}{1 + \left(\frac{R_3}{k} + \frac{R_1}{k_3}\right) s} = p_0 \frac{1}{1 + T\ s}$$

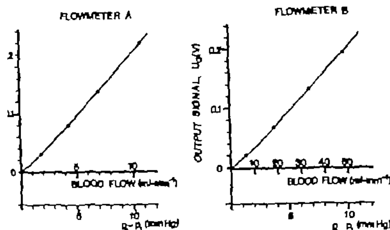
why

$$T_2 = \frac{R_2}{k_2} \quad T = \frac{R_3 + R_1}{k_3}$$

Thus, if k and k are made identical, the relation T = T is valid when R_2 R_1

Design of flowmeter The flowmeter was designed to fulfil the criteria mentioned The $k = k_3$ relation was accomplished by making tube II (outer) and tube III of equal and of the same semirigid polyethylene material The R = R + R relation was accompl by the addition of the small tube inside tube II with a resistance identical to R It is obv that the membrane of the differential pressure transducer must operate without signif displacement, a criterion fulfilled by the chosen piezoresistive transducer (National S LX1601D). Tubes II and III are filled with fluid without air trapping. To avoid deteri tion of the pressure transducer its internal space was filled with the completely inert FC40 (3M Brand inert fluorochemical liquids Chemical Division 3M Company). With design there was virtually no phase lag between p and p_3 (Fig. 1), as evidenced by the abs of arterial pulse pressure oscillations at zero flow during distal artery occlusion. T transducer was hermetically encapsuled and thermoisolated to avoid damage by con with electrolytes and interference by external temperature variation.

The magnitude of the resistance in tube I R (and hence, also R_3) can be varied w a wide range. The value chosen depends on the range within which blood flow is measured, on the upper limit of the arterial blood pressure fall across tube I which can accepted, and on the sensitivity and accuracy of the differential pressure transducer



...strates one recording of the relation between blood flow and the output signal (U_Q) from the transducer for two different flowmeter resistances. Flowmeter pressure fall ($p_a - p_1$) at different ... is illustrated as ... second abscissa. Flowmeter A represents ... tube of 85 mm in length and 1 1 mm ... diameter while flowmeter B represents a tube of 85 mm in length and 1 75 mm in internal di-

...e as small as possible to limit the arterial pressure fall across the flowmeter. With ...ent design of the chosen differential transducer flows within the range from 0 ...l/min can be reliably recorded for an arterial pressure fall across the flowmeter ...to 10 mm Hg.

..., panel A and B, shows calibration curves for two flowmeter types, one with relatively ... designed for measurements in the low flow range (length of tube I 85 mm, internal ...r 1 1 mm) and another with smaller R for larger flows (length of tube I 85 mm ...75 mm). Blood flow (for calibration purpose measured with a graduated cylinder) ...ted versus the output signal (U_Q), from the differential transducer. Along the ...s is also plotted the pressure difference across tube I ($p_a - p_1$). As can be seen, linearity ... satisfactory. This fact also indicates that viscosity changes of the blood within these ... of velocity were insignificant. The present flowmeter design gives a baseline drift/ ... 2% of full scale deflection corresponding to a maximal pressure fall of 10 mm Hg ... the flowmeter at standardized conditions with constant ambient transducer tem...re and constant blood viscosity. Flow calibration including zero flow calibration is ...sted to be tested once every hour.

The vascular resistance meter

...lar resistance is determined by the ratio of mean perfusion pressure and mean blood ... Total vascular resistance in a peripheral vascular bed is thus measured as: (mean ...ial inflow pressure–mean venous outflow pressure)/mean blood flow. Mean perfusion ...ure can be readily obtained from the filtered output signal of a differential pressure ...sducer and mean blood flow from the filtered output signal of an arterial flowmeter

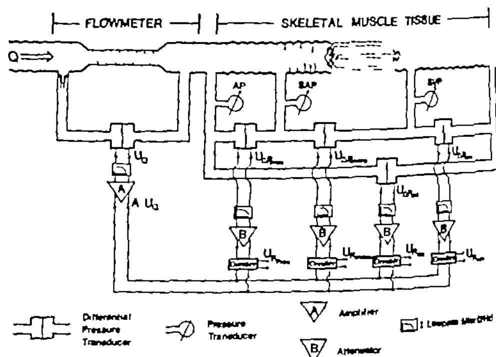
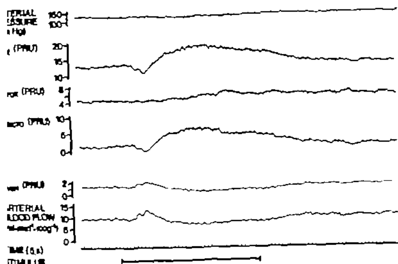


Fig. 3 Illustrates a principle scheme for vascular flow resistance meter when used on skeletal recording t tal resistance ($U_{R tot}$), proximal rterial resistance ($U_{R prox}$), microvascular resistance and large venous resistance ($U_{R ven}$) The scheme also includes continuous registration of flow and tissue pressure fall as B $U_{\Delta P}$ as well as arterial pressure (AP), small artery pressure (SAP) vein pressure (SVP) and enous pressure (VP).

Continuous measurements of vascular resistance can be obtained by feeding these two into an electronic analog divider

In the present resistance meter mean perfusion pressure was measured with a Semic. LX1601D differential pressure transducer and mean blood flow with the meter described in the foregoing, while vascular resistance was obtained with the Analog Devices 532 KD divider Since the voltage ratio of the two input signals electronic divider circuit must never exceed one, the present system requires appr attenuation of the pressure signal and/or amplification of the flow signal.

Fig. 3 Illustrates schematically how the flowmeter and the resistance meter can for the measurements of total and segmental resistances in the vascular bed of the skeletal muscles in the cat The flowmeter is inserted in the cognate artery for contin cording of arterial blood flow Arterial inflow pressure (AP) small artery pressure small vein pressure (SVP) and venous outflow pressure (VP) are monitored from ind catheters as described elsewhere (Grände, Lundvall and Mellander 1977 Grän Mellander 1977) and recorded by Statham transducers. The differential pressure tran provide signals for the pressure drops (ΔP) across the whole vascular bed (AP–VP) the proximal arterial vessels (AP–SAP) across the microvessels (SAP–SVP), and the large venous vessels (SVP–VP) The blood flow signal and the differential signals are fed into the analog dividers to provide continuous recordings of resistance in the whole vascular bed (R_{tot}), in the proximal arterial vessels (R



Polygraph tracing (Grass Polygraph) of effects on arterial pressure, arterial blood flow total s (R_{tot}), proximal resistance (R_{prox}), microvascular resistance (R_{micro}) and large venous resistance t local infusion of norepinephrine (1 0 μg min⁻¹ kg⁻¹) on sympathectomized lower leg skeletal (weight 30 g) of cat, with the experimental approach illustrated in Fig. 3.

ssels (R_{micro}), and in the large venous vessels (R_{ven}) as shown in Fig. 3 Fig. 4 ties how these different vascular resistances are influenced by an infusion of nor hrine.

libration of the flowmeter is performed as described above. Calibration of the total egmental pressure drops is made with the aid of the Statham transducers. Strict al zeroing of all differential pressure transducers at zero differential pressure must ade.

Comments

differential pressure principle for blood flow measurement has been applied in several ous flowmeter designs (.g de Burgh Daly 1926, Lawson and Holt 1939 Ueno and naka 1955). The very small mean pressure gradient over the flowmeter created during flow measurements has been very difficult to record exactly with the so far described es (e.g mechanical, optical, or strain gauge manometers). There have also been rent problems with alinearity phase lag of pulse pressure etc in the previous instru- ts. For these reasons the method has become of little practical use in biological research. ecent developments in the field of piezoresistive pressure transducers, especially the rid IC type (National Semiconductor Corp.), offers the possibility of precise and able measurements of minute differential pressures. The present flowmeter in which h a transducer was used and in which the problems with pressure phase lag were largely rcome, seems to provide a means for reliable and linear recordings of mean blood even in the small flow range. The flowmeter can be designed as small compact unit, s relatively inexpensive, its baseline drift is small and its operation largely independent

of accidental movements. Since the flowmeter is of the cannulating type, antic must, however be administered There is, further an inherent pressure drop a flowmeter in relation to flow which therefore interferes with arterial pressure b disadvantage can be minimized by choosing a small fixed resistance of the flowm (Fig. 2)

The flowmeter has proved to be very suitable as a component in the described r recorder which, in turn, offers the possibility of detailed and reliable continuous r recordings in various vascular beds of the peripheral circulation.

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...cteristics of static and dynamic regulatory mechanisms in myogenic microvascular control

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Abstract

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...described static and dynamic components in myogenic microvascular control (Grände, Lund- ...ander 1977) were analysed in this study with regard to their stimulus-effector characteristics ...icrovascular resistance responses in the sympathectomized vascular bed of skeletal muscle ...l during graded changes of vascular transmural pressure (P_T) applied at different rates (dP_T/dt) from 7.5 to −7.5 mmHg/s. The dynamic microvascular resistance responses, developing ...hase of changing P_T, were pronounced and distinctly graded in relation to the magnitude of ...imulus, both with regard to amplitude of resistance response and rate of resistance change per $R_{...}/dt$). The static responses, revealed in the steady state phase of constant increased P_T, ...latively small and graded in relation to the amplitude of the P_T increase. Rate-sensitivity in ...e myogenic control was bi-directional, eliciting excitatory effects (constriction) in response to ...l inhibitory effects (dilation) in response to negative, values of dP_T/dt. The dynamic constrictor ... given dP_T/dt stimulus increased with increasing amplitude of P_T and, thereby increased dura- ...dynamic stimulus. This effect might be explained by successive activation of myogenic receptor ... different thresholds. The described rate-sensitivity in the myogenic control system seems to im- ...prove stability and sensitivity and thereby can contribute efficiently to well-adapted and re- ...vascular adjustments.

... Myogenic control, local vascular control, myogenic receptor-effector characteristics, rate- ...transmural pressure change, muscle microcirculation, autoregulation, servo-control

...e vascular reactivity has in the past been discussed almost exclusively in terms of ...s constrictor/dilator responses which develop upon a given steady state increase/ ...in vascular transmural pressure. Such steady state effects can be considered to be ...t of a static myogenic stimulus related to the amplitude of the transmural pressure. Recent *in vitro* and *in vivo* studies have demonstrated, however the existence of an ...al and highly effective dynamic component in the myogenic vascular control. Most ... this was evidenced by *in vitro* observations of electrical and mechanical activity in ... single-unit vascular smooth muscle when exposed to a given passive stretch or ...ing applied at various rates of length changes, dL/dt (Johansson and Mellander

1975) A strong static stimulus (steady state increase of length by 40%) was shown to ed only moderate excitatory effects in terms of a 10–15% increase in spike discharge and attendant increase in active force. The dynamic stretch stimulus, operating during the pe- of increasing muscle length was much more effective causing graded excitatory which at high dL/dt values were up to 20 times greater than those evoked by the s stimulus. Passive shortening at different rates caused graded inhibitory responses below t control level, complete inhibition of electrical and mechanical activity being high negative test values of dL/dt. Further analysis suggested that the dynamic recep response was more closely correlated to the rate of change of passive tension (dT/dt) t to dL/dt (Sigurdsson, Johansson and Mellander 1977)

Circumstantial evidence for the presence of a dynamic rate-sensitive component of myogenic control *in vivo* was derived from observations of increased vascular tone upon changes of pressure or shift from steady to artificial graded sinusoidal pressure pulse in the kidney (Basar and Weiss 1969) and upon shift from steady to normal pulsatile pe sion in skeletal muscle (Mellander and Arvidsson 1974) More direct evidence for concept was recently obtained in a study of total and segmental vascular resistance resp in skeletal muscle to standardized transmural pressure stimuli (Grände, Lundvall and lander 1977) These stimuli were induced by exposing all parts of the vascular bed to a change of vascular transmural pressure applied at two distinctly different rates, one and one low The resistance vessels showed clearcut active constriction upon trans pressure increase and active dilation upon pressure decrease. These responses were espe pronounced in the microvessels where a distinct two-component effector response obtained. The initial component, developing during the period of changing pressure, much larger when evoked by high than low rate of transmural pressure change, str indicating that the response was rate-dependent. The subsequent steady state compo revealed during the period of constant increased pressure, was small and rate-indepen

These data indicate that the vascular bed of skeletal muscle is responsive to static as dynamic myogenic stimuli The dynamic responses were almost exclusively confin the microvessels, though reflected also in the total vascular resistance of the region, wh the proximal arterial vessels seemed to be dominated in their control by static myo stimuli. The large venous vessels appeared to lack myogenic reactivity

Little is known about the mode of operation of the myogenic vascular control syste its cellular mechanisms. Apparently the system is more complex than previously bel in view of its recently described rate sensitivity Further progress in this field requires detailed information about the myogenic stimulus-receptor-effector characteristics attempt was made in the present study to define some of these characteristics with r to the static and dynamic components in myogenic microvascular control

Methods

Seven cats, b.wt. 2.8–3.6 kg, were used in the study They were anesthetized with chloralose (50 mg/kg and urethane (100 mg/kg b.wt) after induction with ether Body temperature was kept at 38 ± 0.5°

The observations were made on the acutely sympathectomized vascular bed of the lower leg musc preparation and the experimental set up were basically the same as descr pre

ll and McNamder 1977). In brief, arterial blood flow to the region was recorded continuously after cation (750 I U./kg b t.) with pressure gradient flowmeter (Grände and Borgström 1978) l as short circuit placed between the femoral and the popliteal artery This flowmeter device s accurate registration of mean arterial blood flow Venous outflow of blood from the region was d to the jugular vein via catheter inserted in the popliteal vein. Mean arterial inflow pressure (AP), tery pressure (SAP), small vein pressure (SVP) and venous outflow pressure (VP) in the muscle were monitored continuously with the use of Statham transducers (for technical details see Haddy 954, Grände *et al.* 1977). This experimental approach, combined with plethysmographic recordings able transcapillary fluid movements and capacitance responses, can permit reliable estimations of vascular resistance in the region and of segmental resistances in the "proximal arterial vessels microvessels and the "large venous vessels" according to the definitions given in our previous tions (Grände *et al* 1977) In the present study only total and microvascular resistances were ed, but the technique was here refined so as to provide continuous resistance recordings ith the use trustal pressure transducers and electronic divider circuits (Grände and Borgström 1978). Total r resistance in the muscle region as represented by (AP VP)/arterial flow and "microvascular" nce by (SAP–SVP)/arterial flow in these recordings, neglecting the effects of reduced passive vascular ance/recoil and net transcapillary fluid movement on segmental blood flow Since such effects hardly affect blood flow in the precapillary vessels hich are the myogenically reactive ones and here the resistance resides (see Results), the inherent error in the total and microvascular" resistance de- ations is small and, if anything, will lead to an underestimation of the described active dynamic sitic responses in these vessels (see further Discussion).

en surgery and vascular cannulations were completed, the isolated muscle preparation was placed in pressure-controlled plethysmograph (38°C) filled with Tyrode's solution. The entrance of the plethys- aph, through hich the arterial and venous cannulas passed, was closed hermetically with the aid of ed silicone rubber (Medical Elastomer Dow Corning). The fluid in the plethysmograph was in com- cation via tubing ith an open reservoir placed outside the plethysmograph (Grände, Järhult and nder 1974) Recorded hydrostatic fluid pressure in the plethysmograph could be adjusted to desired l by varying the height of the reservoir in relation to the muscle preparation. Fluid displacements be- the plethysmograph and the reservoir (recorded gravimetrically) represent changes of tissue volume. e control state, plethysmograph pressure was set at the atmospheric level and VP was adjusted to a (~5 mmHg) creating normal transcapillary fluid equilibrium. By lowering the reservoir the plethys- raph pressure, and hence tissue pressure, could be decreased. In this way all parts of the muscle vascular could be exposed to graded increases in vascular transmural pressure (P_T) of different amplitudes, and Pressure changes were applied at different rates (dP_T/dt) over wide range Return of the reservoir to control level permitted declines of vascular transmural pressure at the same graded rates All measured meters were recorded on Grass polygraph. Resistance recordings were stored on magnetic tape ulberg Instrumentation Recorder 109) for later replay on Rika Denki recorder (to avoid curvilinear rtation). Spread of data is given as S E.

Results

animals rested for at least 30 min after completion of the experimental preparation to rmit recovery from the surgical trauma. Blood flow the different pressures and vascular istances in the acutely sympathectomized skeletal muscle region were followed contin- sly throughout the expt. during which the whole vascular bed was exposed, at intervals, a change of transmural pressure (P), accomplished by alteration of extravascular pres- re (see Methods). In one series of expts., P_T was always increased by a total of 30 mmHg ove the normal control level, but this pressure change was applied at different rates in the /dt range from 0.32 mmHg/s to 7.5 mmHg/s. In another series, the amplitude of the P crease was varied as well. I each observation period, P_T was first increased to the high vel at given rate, then maintained constant at this plateau for about 2 min, and finally turned to the control level at the same given rate (see Fig. 1 upper panel). In this way

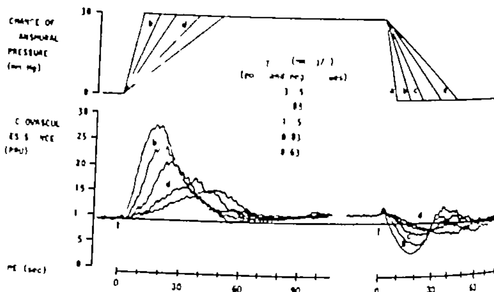


Fig. 1 My genic microvascular resistance responses in sympathectomized skeletal muscle in the cat by increase and subsequent decrease in vascular transmural pressure, P_T, by 30 mmHg applied at 5 diff. rates, dP_T/dt, from 0.63 to 3 75 mmHg/s. The original consecutive resistance recordings are reproduced on top of each other Note the pronounced dynamic constrictor and dilator responses during the pre cha gi g P_T, graded in relation to dP_T/dt both for positive and negative values, and the compar small static constrictor response in the steady state period of constant increased P_T.

the resistance responses to graded dynamic transmural pressure stimuli, both positive negative values of dP_T/dt could be studied ("dynamic" response) as well as the resp to constant increased P_T ("static" response) These effects were compared with the resp values in the control period preceding each experimental intervention. The interval tween consecutive tests of this type was long enough to permit recovery of vascular In some expts., the vascular bed was first exposed to the highest rate of pressure ch and then to gradually lower ones in the others the sequence was reversed, but the did not influence the results.

Although the experimental approach used (see Methods) permits detailed observation total and segmental vascular resistances in the muscle, emphasis will here be placed on responses of the "microvessels" since, as mentioned, these are the ones which exhibit cut reactivity to dynamic myogenic stimuli (Grände *et al* 1977) Microvascular resistance as recorded in this study comprises that of small arterial, capillary and small venous ves (see further below)

Fig. 1 illustrates, in original tracings from one representative experiment, how mic vascular resistance in skeletal muscle was influenced by a given total increase in P_T of mmHg applied at 5 different rates (dP_T/dt 0 63–3 75 mmHg/s) and by subsequent decre in P_T back to the control level at the same given rates. To permit direct comparison results and to limit space, the consecutive original recordings have been reproduced on of each other in such a way that the starting points for increase and decrease in P_T, respectively coincided on the time scale (see arrows below the tracings and graphical illustration of induced P_T change) Control microvascular resistance before pressure elevation averag 9.2±0.3 PRU (mmHg ml min 100 g tissue ⁻¹) in these 5 observ tions.

e of P in each test led to the following general pattern of microvascular resistance (Fig. 1). Within a few seconds after the onset of the P_T rise, resistance started to and in an almost linear fashion reached a peak or transient plateau shortly after attained the final constant level of 30 mmHg above the control value. Resistance lined again, approximately in an exponential manner in spite of the fact that P_T intained constant at the high level. Some 40–60 s after the peak, resistance stabilized dy state level averaging only 1.8 ± 0.2 resistance units (PRU) above the initial control When P was decreased microvascular resistance, after a small and transient (passive) ined to a value clearly below the control level. The subsequent recovery of micro- r tone was characterized by an overshoot and/or an oscillatory behaviour before ce stabilized at its initial control value. The described changes of microvascular ice were caused by simultaneous alterations of SAP SVP and blood flow according same general pattern of response as described in detail in our previous publication le et al. 1977).

resistance data in Fig. 1 demonstrate the existence of a clearcut two-component constrictor response in the microvessels to increased P_T. The initial resistance com- , developing during the phase of increasing pressure and, hence, in all probability to the dP_T/dt stimulus, was large and transient in nature, while the second component nall, though maintained throughout the following period of constant increased pres- Decrease of P back to the control level caused clearcut active dilator responses during se of declining pressure (negative dP_T/dt) before resistance stabilized at the control The magnitude of the second component was related to the amplitude of the P_T in- over a wide range (see Fig. 4).

he constrictor and dilator responses were completely absent in the papaverine-treated, nally dilated vascular bed and here reversed to passive vascular distension effects in se to increased, and passive vascular collapse effects, to decreased transmural pres- as described previously (Grände et al. 1977).

e responses in the normal vascular bed, as will be discussed below can be considered myogenic in nature. The results, which confirm and extend our previous observations nde et al. 1977) strongly indicate the presence of rate-sensitive (dynamic) and a static atory mechanism in the myogenic microvascular control. Evidence for this interpreta- as discussed at length in our previous publication. The rate-sensitive mechanism, in ation during the phase of the P change, was bi-directional causing excitatory effects striction) in response to positive values, and inhibitory effects (dilation) in response to tive values, of dP_T/dt (or rather some receptor modality indirectly related to dP_T/dt). static myogenic mechanism, revealed during the steady state of constant increased P quantitatively much less effective in evoking microvascular constriction.

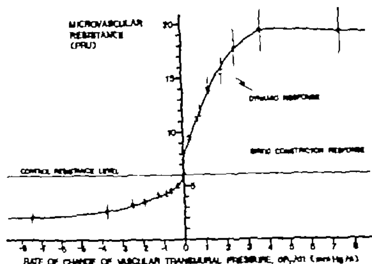
he results in Fig. 1 demonstrate, in particular that the magnitudes of the dynamic strictor and dilator responses were distinctly graded in relation to the magnitude of dP_T/dt hin the range analysed. When P was increased at the rate of 3.75 mmHg/s, microvascular stance thus increased by 18.5 resistance units (PRU) above control the corresponding ne at the low rate of 0.63 mmHg/s was only 7 units. The true dynamic resistance effects ght be 1.8 units less than these values, if the whole static constrictor response (1.8 PRU)

can be considered to be fully developed already at the initial peaks. When P_T was dec. at the high (3 75 mmHg/s) and low (0 63 mmHg/s) rates, resistance declined by 6 and units, respectively below the initial control level (thus representing a true dynamic C response) The dynamic constrictor and dilator responses at the intermediate dP_T/dt (Fig 1) ranged between the mentioned extremes. Note also that there was a distinct rel between the rapidity of the resistance response (both with regard to onset and devel, and the dP_T/dt value. The resistance response thus started 1–? s after the comm. of the P_T change at the highest test value of dP_T/dt but was somewhat more delayed at lower rates (Fig. 1) The resistance change per unit time, dR_{micro}/dt, was graded in rel to the dP_T/dt values, both positive and negative (see further below Fig. 3).

The described dynamic resistance responses in the microvessels were reflected qualit and in rough terms also quantitatively in the simultaneously obtained recordings of vascular resistance in the muscle region This finding warrants our previous conc (Grände *et al* 1977) that dynamic reactivity is mainly confined to the microvess steady state constrictor response in the whole vascular bed to constant increased P_T ceeded, however that in the microvessels, a result in agreement with our previous conc that the proximal arterial vessels, like the microvessels, exhibit clearcut reactivity to myogenic stimuli

Seven series of observations of the type illustrated in Fig. 1 were performed. Fig 2 sum marizes the results in terms of the average dynamic microvascular constrictor and di responses to dP_T/dt stimuli over the entire range investigated (from +7.5 mmHg/s to mmHg/s) The average static constrictor response to the standardized increase of P_T 30 mmHg is also illustrated It can be seen that the microvessels exhibited smoothly gr dynamic constrictor responses to excitatory dP_T/dt stimuli (pressure increase) in the range of test with a maximum reached at a dP_T/dt value of about +4 mmHg/s. At h rates the resistance curve levelled off Similarly there was a graded active microva dilator response to inhibitory dP_T/dt stimuli (pressure decrease), maximal dilator being reached at dP_T/dt values of about −4 mmHg/s. Control microvascular resistance normal P_T in this material averaged 6.0 ± 0.9 PRU corresponding to an average SAP 51 ± 3 mmHg, SVP of 14 ± 2 mmHg and blood flow of 6.0 ± 0.2 ml min 100 g tissue The steady state (static) constrictor response to constant increase of P_T by 30 mmHg control averaged $1.4 \pm 0.$ PRU In constructing the diagram of Fig. 2, we have ass that the static constrictor response was fully developed at the peak of the initial constr component. This implies that the intersect of the dynamic constrictor response curve on ordinate coincides with the static constrictor value, and that the dynamic dilator resp curve starts from the initial control resistance level

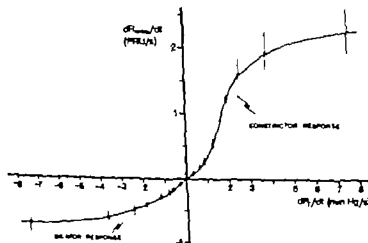
The results in Fig. 1 indicated that the microvascular resistance change per unit ti dR_{micro}/dt, was graded in relation to the dP_T/dt value, which might be another import aspect of dynamic myogenic regulation. This relation was therefore analysed in gre detail by determining dR_{micro}/dt at points where the slopes of the constrictor curves appear to be virtually constant (*cf* Fig. 1) The present deduction thus refers to the points wh resistance in all cases exceeded the control value by 3 PRU The corresponding analysis the dilator curves (which were curvilinear) was made at the p ere they sed



...gram illustrating the average dynamic myogenic microvascular constrictor and dilator responses ...muscle to increase and subsequent decrease in vascular transmural pressure (P_T) by 30 mmHg ...different rates in the dP_T/dt range from 7.5 to 7.5 mmHg/s (n = 7). The average static com...ponent to constant increased P_T by 30 mmHg above the control level is also shown.

...resistance line. Fig. 3 summarizes the results for the whole material (7 expts). It ...ems that dR_{micro}/dt at the points of measurement was graded in relation to positive ...ative values of dP_T/dt over a wide range.

...lts in Fig. 1 demonstrated that the microvascular constrictor response to dP_T/dt ... level off even when the dynamic stimulus was quite prolonged, but continued to ... as long as the vascular bed was exposed to the stimulus. The peak response was



... Diagram illustrating microvascular resistance change per unit time (dR_{micro}/dt) during increase ...subsequent decrease in vascular transmural pressure (P_T) by 30 mmHg at different rates in the dP_T/dt ...from 7.5 to −7.5 mmHg/s (n = 7). For details see text.

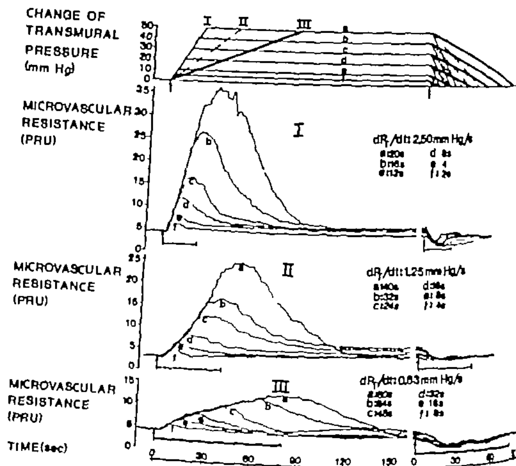
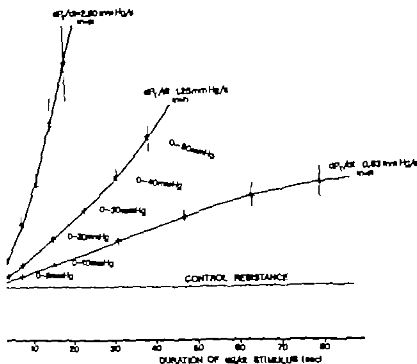


Fig. 4 Myogenic microvascular resist nce responses evoked by graded increases in vascular trans... pressure, P_T, by 5 to 50 mmHg above control applied at three different rates (see key I-III) and ... sequent decreases in P_T t the same rates. The original consecutive resistance recordings are ... on top of each other Not that the resistance responses at given dP_T/dt value appear to be ... relation to the amplitude of the P_T change and/or the duration of the dP_T/dt stimulus (see key ... the curves indicates the longest duration of dP_T/dt stimulus in each series). The static, rate-independent ... strictor responses are graded in relation t the amplitude of the P_T increase in the range from 5 to ...

thus reached shortly after the cessation of the dynamic pressure change which, in ... prolonged dynamic stimuli, seems to be an unusually long smooth muscle "contraction time" (> 48 s) This finding might have several explanations (see Discussion).

In an attempt to elucidate this problem microvascular resistance was observed when ... vascular bed was exposed to P_T changes of different amplitudes in the range from 5 ... mmHg above control applied at three different rates, 0 63 1.25 and 2.5 mmHg/s (Fig ... This approach also implied variation of the duration of the dynamic stimulus over a ... range up to 80 s (see key in Fig. 4)

It can be seen from this figure that the microvascular constrictor responses (plotted ... top of each other as in Fig. 1) at each dP_T/dt value followed an almost identical slope ... their development, reached their peaks not until shortly after cessation of the applied dP_T... stimulus, and were quantitatively graded in relation to the amplitude of the P_T increase (... the duration of the dP_T/dt stimulus) Calculated dR_{micro}/dt for each series (I-III) varied ... direct relation to the dP_T/dt value (cf Fig. 3) Note also the distinct influence of dP_T/dt ...



...pled data for the peak constrictor responses of the micro-vessels evoked by increases in vascular ...ressure as shown in Fig. 4. The constrictor responses to the three different dP_T/dt stimuli ...versus the duration of the dynamic stimulus which also implied ... simultaneous change of the ...de (see dotted lines). The dynamic myogenic constrictor response seems to be influenced by ...ude of the dP_T/dt stimulus and, indirectly by its duration, and by the amplitude of the P_T ...rther explanation see text).

...itude of the peak constrictor response at each level of P change (5 10, 20, 30, 40, ...mmHg). The delay from the onset of the dP_T/dt stimulus to the start of the constrictor ...was almost identical at each dP_T/dt value, but was shorter the higher the dP_T/dt value. ...eement with the results in Fig. 1 resistance declined to steady state levels during ...d of constant increased P_T, and these static responses were graded in relation to the ...de of the P increase within the range from 5 to 40 mmHg (Fig. 4). When P was ...d as much as 50 mmHg, the active static constrictor response seemed to just barely ...out the inherent passive vascular distension effect, implying an approximate return ...tance to the control level. The steady state response at each P level was of similar ...de in panels I–III (like in the whole material) strongly indicating that it was in...ent of the preceding dP_T/dt stimulus. The static transmural pressure stimulus, when ...l (40 mmHg), increased resistance by no more than some 2 PRU above control ...nce, the static response was quite small compared to the up to 15 times larger dynamic ...e (Fig. 4). The effectiveness of the dynamic stimulus may be illustrated by the fact ...e weak dP_T/dt stimulus of 0.63 mmHg/s at such a small P increase as 5 mmHg ...l a distinct dynamic constrictor response (Fig. 4, III f).

Decrease of P_T from any level applied at any rate depicted in Fig. 4 (right side) evoked active dilator responses in the microvessels. Mention should be made that dilator responses, in contrast to the constrictor effects, often reached their maxim[illegible] the cessation of the inhibitory dynamic stimulus (*i.e.*, when the duration of the st[illegible] exceeded about 12 s). This difference, as will be discussed, may have important func[illegible] implications.

Fig. 5 shows compiled data for the peak constrictor responses from the whole se[illegible] experiments of this type (*cf.* Fig. 4). The microvascular constrictor responses to the different dP_T/dt stimuli are here plotted versus the duration of the dP_T/dt stimulus [illegible] also involves a simultaneous change of the P_T amplitude, illustrated by the dotted [illegible] It can be seen that the peak constrictor response, at any of the three dP_T/dt values, [illegible] with increasing duration of the dynamic stimulus and/or increasing amplitude of t[illegible] rise. The responses were also clearly graded in relation to the magnitude of dP_T/d[illegible] data in Fig. 5 can be considered to reflect dynamic reactivity mainly since a static co[illegible] possibly included in the peak response, is comparatively small.

It thus appears from Fig. 5 that the dynamic constrictor response is influenced no[illegible] by the magnitude of the dP_T/dt stimulus (*cf.* Fig. 2) but also in some way by its d[illegible] and/or the amplitude of the P_T change (see Discussion). The relative quantitative [illegible] of the latter two factors is difficult to separate from each other. The following e[illegible] from the diagram might illustrate the mutual interrelationship between these three va[illegible] At a fixed P_T amplitude of 30 mmHg the dP_T/dt stimulus of 1.25 mmHg/s, lasting f[illegible] evoked a constrictor response of roughly the same magnitude as the dP_T/dt stim[illegible] 0.63 mmHg/s, lasting for 48 s. The dP_T/dt stimulus of 2.5 mmHg/s at the same P_T am[illegible] was, however, much more effective, despite its short duration of 12 s. It thus appea[illegible] the magnitude of dP_T/dt, as expected, is the major determinant of the dynamic resp[illegible]

All observations in this study were made on the acutely sympathectomized vascul[illegible] to avoid adrenergic influence. There is some evidence in the literature, however, that co[illegible] tor effects to raised transmural pressure, in part, might be mediated by a *local* adr[illegible] nervous mechanism (*e.g.* Henriksen 1976). To investigate this possibility the de[illegible] static and dynamic microvascular effector responses were studied in several expts. [illegible] and after local α- and β-adrenergic blockade with phenoxybenzamine (15 mg kg⁻¹ [illegible] tissue, *i.a.*) and propranolol (1 mg kg⁻¹ *i.a.*), respectively. Effective adrenoceptor bl[illegible] was achieved, as evidenced by complete abolition of adrenergic constrictor and dil[illegible] sponses to large amounts (10 μg kg⁻¹ *i.a.*) of noradrenaline and isoprenaline. In [illegible] were the microvascular constrictor and dilator responses to transmural pressure [illegible] significantly attenuated by the adrenergic blockade, which strongly indicates th[illegible] described vascular reactions were myogenic in nature.

Discussion

The "microvessels" investigated in this study were delineated by the sites along the v[illegible] tree from which small artery pressure and small vein pressure, via collaterals, were mea[illegible] The dimensions of these vessels on the arterial side can be rou[illegible] from d[illegible]

ns pressure and diameter measurements in the muscle microvessels reported by d Zweifach (1975). Our average value for small artery pressure of 51 ± 3 mmHg rol period then indicates that this pressure was monitored from arterial vessels in f 15 μm and that the described vascular reactions therefore were mainly confined vessels of this size and smaller. Minute venous vessels were also included in the nts of microvascular resistance.

sent experimental approach to determine segmental vascular resistance bears a the basic principle for determination of pre-/postcapillary resistance advanced heimer and Soto-Rivera (1948). In the present study blood flow through all sec e vascular tree, especially during the period of phasic shift in transmural pressure, r not necessarily exactly equal to arterial inflow owing to effects of passive vascular or recoil ("capacitance response") and net transcapillary fluid movement. Such e certainly encountered on the highly distensible venous side, and "large vein " in this type of investigation can only be approximately determined as (SVP VP)/ utflow (or instead of venous outflow arterial inflow minus recorded net fluid t and capacitance effects) as done in our previous study (Grände et al 1977). If ease is very rapid, SVP might even fall below VP for a few seconds and reversal in the veins would then have occurred unless it was prevented by the venous lood flow through the myogenically reactive arterial vessels, on the other hand, considered to be quite close to arterial inflow since passive distension caused sic P increase is small here (Grände et al. 1977) and in the overall microvessels negligible to judge from the absence of an initial decline of microvascular resist ring the period of increasing P (Fig. 1). Note in this context that if flow through illaries and minute veins in fact is somewhat smaller than arterial inflow during , and somewhat larger during decrease, of P we have, if anything, underestimated cribed active reactions in the microvessels to transmural pressure stimuli, but the t error must be relatively small.

plethysmographic technique permitted all parts of the sympathectomized vascular skeletal muscle to be exposed to strictly graded changes of vascular transmural pres- alteration of extravascular (plethysmographic) pressure without affecting the venous pressure difference across the whole vascular bed. Vascular reactivity to ified transmural pressure stimuli could thus be studied without significant inter : from the local chemical metabolic control system, since the induced changes of flow were quite small (for more detailed discussion see Grände et al. 1977 cf Johnson 976). The described resistance responses to transmural pressure stimuli therefore most were myogenic in nature, a conclusion further corroborated by the facts that they ally maintained after adrenoceptor blockade but completely abolished after papaverine ostration.

our previous publication (Grände et al. 1977) evidence was obtained which strongly ted the presence of a static as well as a dynamic component in myogenic microvascular ol, an interpretation supported by the recent in vitro studies on vascular smooth muscle oned in Introduction.

e present results provide additional evidence for this concept. The dynamic micro- ...

vascular resistance responses were thus found to be distinctly graded in relation to the magnitude of the dynamic stimulus, dP_T/dt, both for positive and negative values (Fig. and the static resistance responses, though small, were graded in relation to the extent of the applied P_T change over a wide range (Fig. 4) The microvascular resistance change per unit time (dR_{micro}/dt) was also directly dependent of the magnitude of the dP_T/dt stimulus both for positive and negative values (Fig. 3) The dynamic myogenic control system is bi-directional evoking constriction in response to excitatory stimuli (positive values of dP_T/dt) and dilation in response to inhibitory stimuli (negative values of dP_T/dt).

The *in vitro* studies on isolated single-unit vascular smooth muscle mentioned in Introduction (Johansson and Mellander 1975 Sigurdsson *et al* 1977) may help to explain the cellular mechanisms behind these excitatory and inhibitory myogenic microvascular responses. Those investigations demonstrated the existence of clearly graded dynamic contractile myogenic responses to passive stretch at various rates (different dL/dt values) and that these responses were caused by graded facilitation of spike discharge primarily generated by "pacemaker" cells. Passive shortening at different rates caused relaxation by graded inhibition of normal pacemaker activity The curve relating electrical (and mechanical) smooth muscle responses to graded dL/dt values in these *in vitro* studies (Johansson and Mellander 1975 Fig. 3) shows conspicuous similarity to the present curve relating the vascular effector response to dP_T/dt (Fig 2) and to that relating dR_{micro}/dt to dP_T/dt (Fig It seems very likely therefore, that the dynamic resistance responses *in vivo* are mediated via a similar myogenic pacemaker mechanism. If so it appears that the dynamic stimulus via facilitation or inhibition of spike discharge is a determinant of both the magnitude of the dynamic vascular effector response and its rate of development

Dynamic stretch *in vitro* especially when performed at high dL/dt, evoked an afterdischarge and an attendant contractile response for some time after the cessation of the dynamic stimulus, apparently due to somewhat prolonged deformation of the receptor unit (Johansson and Mellander 1975). Such an afterdischarge might at least partly explain why the peak microvascular constrictor responses were not reached until some time after the end of the dP_T/dt stimulus (Fig 1).

A constant dynamic stimulus, when prolonged would be expected to evoke a plateau constrictor response at the time the receptor-effector unit is maximally activated. A tendency of such an effect was occasionally noted, but only when the dP_T/dt stimulus lasted for or more (Fig. 4 III, Fig. 5 lower curve) This, as mentioned, seems to be an unusually long "contraction time" for smooth muscle The gradually increasing constrictor responses during prolonged dynamic stimulation at increasing P_T (Fig. 4 and 5) can only to a small extent be ascribed to a gradually increasing static response It could be explained by very slow propagation of spikes from the receptor unit to a gradually increasing number of effector cells, but this seems unlikely in view of reported electrical propagation velocities of no less than 0.5–3 cm/s in other types of myogenically active vascular smooth muscle (Ljung & Stage 1970) The dynamic receptor might in reality be sensitive to the rate of wall tension change, $d\sigma/dt$ (see below). Wall tension can be calculated to increase gradually during rising P_T (by more than 100% at the peak of curve I a, Fig. 4) despite the counteracting effect of active lumen reduction. $d\sigma/dt$ is more difficult to est t but rough deductions

ents that it also might increase gradually and, if so, could be a stimulus for the con- ...ly increasing constrictor response. More likely perhaps, the described phenomenon ... be explained by gradual recruitment of an increasing number of dynamic receptor ...with different thresholds when P_T rises. The fact that resistance increased at an almost ...ant rate for a given prolonged dP_T/dt stimulus at gradually increasing P (Fig. 4) ... to support the latter hypothesis, since threshold phenomena in biological control ...known to give rise to such effector characteristics (Licko 1973). The fact that the ...or responses to negative dP_T/dt often reached their maxima before the cessation of ...dynamic stimulus (Fig. 4) might then simply be explained by a small number of ...hold units in operation at the preceding low level of vascular tone.

...reality the dynamic myogenic receptor is probably not sensitive to dP_T/dt directly ...ight possibly be sensitive to length changes, dL/dt, provided the sensor element is ...led in series with the contractile elements (see Johnson 1974), but perhaps more likely ... sensitive to changes of wall tension, as suggested from *in vivo* and *in vitro* observa- ...s (Johnson and Intaglietta 1976, Sigurdsson *et al.* 1977). The latter authors further ...d that the dynamic myogenic responses to passive stretch were equally pronounced ...er conditions when vascular smooth muscle contraction manifested itself as active ...s ("isometric" contraction) or as active shortening ("isotonic" contraction, like *in vivo*). ... present investigation clearly demonstrates that active constriction and, hence, smooth ...cle shortening *in vivo* does not result in abolition of dynamic myogenic reactivity.

... rate-dependent regulatory mechanism is of great importance in most servo-control ...ems in order to increase rapidity, stability and sensitivity (Milsum and Schultz 1969). ... present data, taken together, indicate that the dynamic component in myogenic micro-...cular control serves such purposes. For proper function, most servo-control systems ...uire a smoothly graded relation between the rate-dependent input signal and the resulting ...put signal, in this case corresponding to the dynamic stimulus and the effector response ...like frequency, resistance change, and/or dR_{max}/dt). Our results indicate that the dynamic ...ogenic microvascular control system also fulfils this criterion (Fig. 2, 3). Rate-dependent ...ogenic microvascular control was further shown to be bi-directional, evoking graded ...nstrictor effects in response to positive, and graded dilator effects in response to negative, ...lues of dP_T/dt. Such characteristics greatly increase the effectiveness of a servo-control ...stem (Milsum and Schultz 1969). Basar and Weiss (1969), however, reached the conclusion, ...on observations on the artificially perfused kidney, of a uni-directional rate-sensitivity ... myogenic control, but the absence of inhibitory dynamic effects in their study might ...ssibly have been due to low vascular tone and reactivity in that particular preparation.

It is possible that there are separate "receptors" for the static and dynamic stimuli in ...myogenic control (like in the highly differentiated muscle spindle of skeletal muscle), an ...pinion tentatively supported by the finding of predominant reactivity to static stimuli in ...e proximal arterial ...essels and predominant reactivity to dynamic stimuli in the micro-...essels (Grände *et al.* 1977). The fact that strong inhibitory dynamic stimuli (high negative ...P_T/dt) were able to cause profound decrease of vascular tone below the control level (Figs. ... 2, and 4 1) shows that they can interfere with the mechanisms responsible for normal ...basal myogenic tone in the microvessels; dynamic stimuli might therefore contribute to

the very establishment of basal myogenic tone in the microvessels, for instance on p[illegible] pressure distension (Mellander and Arvidsson 1974 LaLone 1975). The great sensitivity [illegible] prompt reactivity of the microvessels to dynamic stimuli, demonstrated in this study [illegible] indicate that rate-sensitivity is of fundamental importance in normal microcirculatory [illegible]lation It might here, by adjustments of precapillary sphincters and small resistance [illegible] contribute to various autoregulatory phenomena, for instance to autoregulation of capi[illegible] hydrostatic pressure accomplished by myogenic resettings of the ratio of pre-/postcapi[illegible] resistance (Järhult and Mellander 1974) No doubt, static myogenic stimuli are [illegible]portant for circulatory control (e.g Baez, Laidlaw and Orkin 1974) and might, by [illegible] influence on proximal arterial vessels as well, be of special significance for local regul[illegible] of the overall resistance function in the vascular bed for instance in myogenic autoreg[illegible]tion of blood flow A tentative theory for blood flow autoregulation based mainly [illegible] repetitive dynamic myogenic stimulation of the vascular bed has been presented [illegible] (Mellander 1978)

This study was supported by grant .210-14X from the Swedish Medical Research Council and by [illegible] from the Medical Faculty University of Lund, Sweden.

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Nicotinic acid stimulates prostaglandin synthesis in the rabbit heart without releasing noradrenaline

By

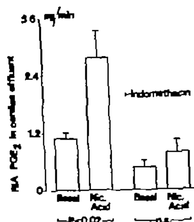
LENNART KAIJSER and ÅKE WENNMALM

Nicotine is a powerful stimulator of prostaglandin (PG) synthesis in the rabbit heart (Wenn-malm and Junstad 1976) The mechanism behind this synthesis stimulation is not known. Nicotine also elicits a short-lasting— explosive"—release of sympathetic neurotransmitter (noradrenaline, NA) in this organ (Löffelholz 1970) Since infusion of NA into the rabbit heart is known to stimulate its synthesis of PGs (Junstad and Wennmalm 1973) a possible mechanism behind the synthesis stimulating action of nicotine would be that it is mediated *via* the NA released. This would imply that the two effects of nicotine—release of NA and stimulation of PG synthesis—are closely associated

Nicotinic acid (NIC), which has the pyridine ring in common with nicotine, has also been suggested as a stimulator of PG synthesis in some different tissues (Andersson *et al.* 197 It was therefore of interest to study firstly if NIC stimulates PG synthesis in the rabbit heart, and secondly if such stimulation occurs in parallel to a release of NA, or alternatively is dissociated from NA release.

Isolated rabbit hearts were perfused according to Langendorff with gassed and heated Tyrode solution. The effluent from the heart was collected and analysed for NA using the trihydroxy indole method. PGs in the effluent were adsorbed on Amberlite XAD-2 and eluted with ethanol After evaporation the residue was quantified using a commercial kit for radio-immunoassay (RIA) of PGE (Clinical Assays Inc.) against a standard curve of PGE_2. NIC and indomethacin were added to the Tyrode solution to produce final concentrations of 2 10^{-4} M and 5 10^{-6} M, respectively Perfusion of the heart was performed in 4 consecutive 10 min periods (I–IV) and effluent for analysis of PG and NA was collected during the last 5 min in each period. During I the heart was perfused with drug-free Tyrode solution and during II it was perfused with a solution containing NIC. During III indomethacin a PG synthesis inhibitor (Vane 1971), was added to the Tyrode solution and during IV both NIC and indomethacin were present in the solution perfusing the heart.

Perfusion with NIC increased the basal outflow of RIA-PGE_2 by more than 150% (Fi compared to the basal outflow No significant outflow of NA was found in the effluent neither at rest nor during perfusion with NIC. Heart rate and contractile force remained unchanged during perfusion with NIC. Perfusion with indomethacin decreased the



Outflow of radio-immunoassayed (RIA) PGE_2 from isolated rabbit heart perfused with drug-free (Basal) solution and with solution containing nicotinic acid (Nic. Acid, $2 \cdot 10^{-4}$ M), in the absence (the two left columns) and in the presence (the two right columns) of indomethacin ($5 \cdot 10^{-6}$ M).

...flow of RIA-PGE_2 by more than 50%. Subsequent perfusion with NIC, in the presence of indomethacin, did not significantly increase the efflux of RIA-PGE (Fig. 1).

Comment

The current data demonstrate that NIC (in the absence of the PG synthesis inhibitor indomethacin) facilitates rabbit heart PG synthesis. Furthermore the data clearly show that stimulation of the PG synthesis by NIC occurs in the absence of release of NA. This implies that NIC stimulates PG formation directly probably by facilitation of the rate-limiting step of this synthesis, which is the mobilization of arachidonic acid from the tissue stores. Since NIC stimulates PG formation by a direct action it seems, by analogy likely that nicotine—chemically closely related to NIC—facilitates PG synthesis via the same mechanism.

The present data support the concept that nicotine and NIC, by a direct action, stimulate the bioformation of PGs. Such stimulation may be referred to the action of the common denominator of these compounds—the pyridine ring—on a receptive function in the myocardial cell membranes.

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The occurrence of somatostatin-like immunoreactivity in the vagal nerves

By

KERSTIN UVNÄS-WALLENSTEN, SUAD EFENDIĆ and ROLF LUFT

Electrical stimulation of the vagal nerves is followed by a release of somatostatin and into the antral lumen of anesthetized cats (Uvnäs-Wallensten *et al* 1977 a). The am of somatostatin released in this way are quite small in comparison to the total antral c of somatostatin The release mechanism is probably not cholinergic since atropine d block the release response (Uvnäs-Wallensten *et al*., to be published). Accordingly, curred to us that electrical stimulation of the vagus might release somatostatin from and not from D-cells In this connection, it is of interest that within the gastroint tract somatostatin-like immunoreactivity has been localized to nervous structures (F *et al* 1975) in addition to in endocrine-like cells. Furthermore, gastrin 17 is present in from vagal nerves, and a gastrinergic nerve plexus, confined to the distal part of th intestine and the colon has been demonstrated by immunohistochemistry (Uvnäs-W sten *et al* 1977 b)

For the above reasons it was investigated whether somatostatin-like immunoreac could be detected in the vagal nerves. For this purpose, pieces of the cervical, thorac abdominal vagi were collected from 6 anesthetized cats, and abdominal vagal specime taken from 2 dogs. The biopsy material was immediately frozen on dry ice. Afte weighed, the frozen nerves were cut into small pieces and boiled for 20 min in water boiling, the volume of the extracts was adjusted to 10 ml and the content of somat determined by radioimmunoassay (Elde *et al* 1977 a, 1977 b) The antibodies used present studies have been further characterized by Arimura (1977).

Somatostatin (80–600 ng/g) was found in the abdominal parts of the vagi from all whereas smaller amounts (0–300 ng/g) were found in the cervical and thoracal parts nerves (Table I) In the dogs, somatostatin-like immunoreactivity (17–40 ng/g) w found in the abdominal section of the vagal nerves (Table I).

At present we cannot state the origin of the somatostatin containing fibres in th Most problably they come from vagal centres in the medulla oblongata The accum of somatostatin in the distal part of the nerves supports this assumption since trans substances tend to accumulate in the distal part of the neurons.

The possible function of the vagal somatostatin containing fibres can only be spe upon. These nerves may be involved in the well known regulat— —tions exerted

Table II. Concentration of somatostatin (ng/g) in different parts of the vagal nerves.

	Part of the vagal nerve		
	Cervical	Thoracic	Abdominal
1	0		90
2	0	20	300
3	0		120
4	10		80
5	50		400
6	100	300	600
1			17
2			40

l nerves on gastrointestinal motility and tone as well as on HCl secretion. In this context, of interest that somatostatin inhibits HCl secretion (Bloom, *et al.* 1975, Le Roith *et al.* Rapth *et al.* 1975, Albinus *et al.* 1975) and gastrointestinal motor functions (Guillemin Blendle and Mattsson 1977). Guillemin demonstrated that somatostatin reduced the ...itude of the contractions of the longitudinal muscle of the guinea-pig ileum caused by ...rical vagal stimulation, and that this effect was mediated by a decrease in the release of ...lcholine, indicating that somatostatin might act as an inhibitory modulator of cho...rgic transmission (Guillemin 1977).

...contrast to somatostatin, gastrin has stimulatory effects on HCl secretion and on ...rointestinal tone and motility. Vizi *et al.* (1973) have shown that part of the myotonic ...ct, exerted by gastrin, *is in fact caused by a release* of acetylcholine from the Auerbach ...us.

...hus both gastrin and somatostatin can influence *the release of ACh from ganglionic cells* ...he gastrointestinal tract (Vizi *et al.* 1973, Guillemin 1977). These findings, together with ...demonstration of gastrin and somatostatin like immunoreactivity in the vagal *nerves* ...Vahs-Wallensten *et al.* 1977 b) suggest that not only cholinergic fibres run in the vagi but ...o somatostatin and gastrin containing fibres, which may exert their actions by changing ...release of acetylcholine from ganglionic cells. It is possible that selective activation of ...he antagonistic vagal fibres may be one of the ways by which the CNS can control gastro...estinal functions, perhaps via modulation of cholinergic transmission.

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Inhibitory effects of noradrenaline and prostaglandin E_2 on neurotransmitter secretion evoked by single shocks or by short trains of nerve stimuli

By

LENNART STJÄRNE

mount of noradrenaline (NA) secreted per nerve impulse, as the result of electrical lation of adrenergic nerves, is highly dependent on the time interval between successive ses (Hughes 1972) as well as on the number of impulses in each stimulus train (Hughes Roth 1974)

e present paper is a preliminary report of studies of different factors modifying the tating effect of repetitive impulses on the secretion of ^{3}H-NA evoked by electrical stimun of the postganglionic sympathetic nerves of guinea-pig isolated vas deferens (Stjärne, published). It describes, firstly an attempt to add to the previously available knowledge erning the facilitating effect of repetitive stimulation, by using the measured secretion H-NA per shock evoked by trains of 5–300 impulses to estimate the secretion caused by gle nerve impulse. Secondly it reports the effect of variation of the calcium level in the um, and of interference with the 2 feed-back control mechanisms normally restricting secretory mechanism (for references see for example Stjärne 1973), on the secretion of NA evoked by a single nerve impulse, as well as on the facilitating effect of repetitive ulses.

nea-pig vasa deferentia, in which the NA stores of the adrenergic nerves had been labelled by preincubation with 2.4 µM ^{3}H-( )-NA, were superfused with modified Tyrode solution (calcium level adjusted 6, 1.8 or 5.4 mM), containing 20 µg/ml sodium ascorbate, 0.6 µM desipramine, 10 µM normetanephrine 1 µg/ml atropine sulphate. The medium was aerated with carbogen gas (93.5 % O_2, 6.5 % CO_2). Temperature was 37°C and flow rate 2.5 ml/min. A Grass S44 Stimulator operated at 90 V 0.3 ms, was used for trical postganglionic stimulation of the extramural nerves, via 2 platinum ring electrodes mounted 8 mm rt around the nerve trunk at its entry into the vas. The frequency of stimulation was 10 Hz. The number shocks in each stimulation period was 300, applied either in one sequence, or subdivided into separate ns of 5 15 or 50 shocks repeated with 10 intervals. The average fractional secretion of ^{3}H NA per shock was determined by dividing the rise in ^{3}H efflux caused by the nerve stimulation in each period with the mber of shocks applied (300) and with the total amount of ^{3}H (essentially equal to total ^{3}H-NA) present the tissue at the time of stimulation. The value for Δt caused by single nerve impulse was estimated extrapolation from the average values for Δt caused by trains of 5, 15 and 50 shocks.

e results are shown in Fig. 1 Under each experimental condition Δt increased with the ngth of stimulus trains. A log log plot of Δt against the length of stimulus trains formed a aight line for the Δt values obtained on stimulation with trains of 5, 15 and 50 shocks.

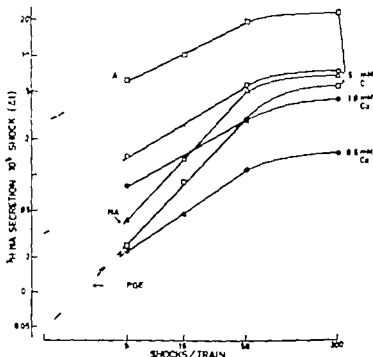


Fig. 1 Log-log plot of the ... secretion of ^{3}H-NA per ... (Δt) as a function of the ... stimulus trains, at ... 5.4 mM calcium. Effects of ... phentolamine alone, of 1 μM ... and of 10 μM phentolamine ... presence of 0.1 μM PGE, ... experiments ... carried at 5.4 mM calcium. Each ... represents the average of ... observations. Broken line ... extrapolation to ... single shock value for Δt

Linear extrapolation from these values was used for tentative estimation of Δt caused b... single nerve impulse. Beyond a train length of 50 shocks a steady state was approached... apparently fully attained with trains of 300 shocks.

On stimulation in normal Tyrode (1.8 mM calcium) repetitive stimulation caused a 14... rise in Δt, from an estimated level of 0.3 10^{-4} of the total neuronal store of ^{3}H-N... stimulation with a single nerve shock, to 4.1 × 10^{-4} at the "fully facilitated" state, (o... stimulation with trains of 300 shocks. Reduction of external calcium to 1/3 (to 0.6 ... depressed Δt, and a 3-fold rise in calcium (to 5.4 mM) enhanced Δt, at all train length... cluding single nerve impulses. These results show that variation in external calcium str... influenced the absolute values for Δt at all train lengths, but did not greatly affe... magnitude of facilitation due to repetitive stimulation during the 9-fold change in ex... calcium (from 0.6 to 5.4 mM) the average value for $\Delta t_{300\ shock\ trains}/\Delta t_{single\ shock}$ was ... tively similar 14.9 ± 4.4 (n = 3).

Addition of 10 μM phentolamine (at 5.4 mM calcium) to remove α-adrenoceptor me... restriction of the secretory mechanism resulted in enhancement of Δt at all train le... The effect was inversely related to train lengths $\Delta t_{300\ shock\ trains}/\Delta t_{single\ shock}$ was redu... 40%, to 7.7 compared with 12.8 at 5.4 mM calcium in the absence of phentolamine.

While addition of 1 μM NA (at 5.4 mM calcium) to stimulate the inhibitory (α... α-adrenoceptors did not significantly alter the "fully facilitated" level of Δt, it did dras... depress Δt caused by a single shock or by short trains of nerve stimuli. $\Delta t_{300\ shock}$... $\Delta t_{single\ shock}$ was enhanced 7-fold, to 90.3 compared with 12.8 at 5.4 mM calcium ... absence of NA. As seen in Fig. 1 the effect of 1 μM NA on Δt evoked by single shoc... equivalent with that of a 9-fold reduction in external calcium.

...ffect of addition of 0.1 µM PGE_2 in the presence of 10 µM phentolamine (at 5.4 mM ...) was also inversely related to the length of stimulus trains. PGE_2 depressed the 'fully ...ed' level of Δt in the presence of phentolamine alone by 76%, but Δt evoked by a ...erve shock by more than 98%. The ratio $\Delta t_{\text{six shock train}}/\Delta t_{\text{single shock}}$ was enhanced from 7.7 with phentolamine alone to 118 with phentolamine + PGE_2.

...resent results show that the level of external calcium influences the absolute value for ...ll lengths of stimulus trains, but not the magnitude of the facilitation caused by ...ve stimulation. By contrast the 'auto-inhibition' of the secretory mechanism triggered ...ogenous NA, and the inhibitory effects of exogenous NA and PGE_2, influence both ...solute levels of Δt at all train length (exception: exogenous NA did not significantly ...e 'fully facilitated' level of Δt on stimulation at 10 Hz, but it has previously been shown ...so at lower frequencies, Stjärne 1973), and the magnitude of the facilitation caused by ...ive stimulation. Their effects were thus inversely related to the length of stimulus ... These findings appear to have practical implications for the interpretation of previous ... obtained in guinea-pig isolated vas deferens. The particularly striking depressing ... of exogenous NA and PGE_2 on Δt evoked by very short trains of stimuli may thus ...in the observation (Ambache and Zaar 1971) that exogenous NA depressed the con... response to nerve stimulation with train of 5 shocks at 10 Hz, as well as the finding ...PGE_2 depressed contractions caused by short trains (5–25 shocks), but less strongly (or ... at all) depressed those evoked by longer trains (50 or more shocks) at 5–50 Hz (Euler and ...rist 1969, Ambache and Zaar 1971, Knoll *et al.* 1975).

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Cerebral sodium noradrenaline interaction: dipsogenic, antidiuretic and natriuretic effects

By

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Intracerebroventricular (IVT) infusions of noradrenaline (NA), other monoamines, acetylcholine, have previously been shown to cause apparent release of antidiuretic hormone (ADH) in the hydrated goat (Olsson 1970 Vandeputte-Van Messom and Peeters ... Several investigators have found that these neurotransmittors may elicit drinking when injected into the hypothalamic region of the rat (cf Fitzsimons 1972). The dipsogenic responses have generally been regarded as due to transmittor effects at various synapses, different, more or less elaborate models of mixed monoaminergic and cholinergic pathways in the cerebral thirst mechanism have been constructed. Another possibility would be that IVT administration of monoamines elicit thirst and ADH-release by acting on ... ular receptors involved in the control of fluid balance The striking sodium-noradrenaline interaction briefly reported here speaks in favour of this possibility

Two female goats (b wt *ca* 35 kg) were used The animals were maintained in positive sodium balance and had free access to water They were supplied with permanent ... iridium cannulas opening into the anterio-dorsal part of the third cerebral ventricle. The construction of the cannula-system and the infusion technique have been described earlier (Åkerlund, Andersson and Olsson 1973) The IVT infusions (duration 20 min, rate ... min) were made in the hydrated animal (90 min after the intraruminal administration of 100 ml/kg of 37°C water) Urine was collected via a retention catheter in 10 min samples for determination of flow rate osmolality [Na], and [K] The following IVT infusions were made at random order with a minimum interval of two days a) NA (l-noradrenaline bitartrate 30 pmol/kg min) in either hypertonic (0.3 M) NaCl, isotonic (0.15 M) NaCl, or isotonic (0.3 M) d-glucose, and b) merely 0 3 M NaCl Five experimental series including the four kinds of infusion were made Two additional experimental series were also performed in which the dosage of NA was reduced to 7.5 pmol/kg min Figures presented represent Mean ± S E.

Thirst All infusions of NA/0 3 M NaCl ($n = 7$) induced cumulative drinking in spite of the fact that the goats were hydrated at the start of the infusion The latency time for drinking in response to the larger dosage of NA in hypertonic NaCl ($n = 5$) was 9 ± 1.5 min with a total water intake of $1\,400 \pm 80$ ml Also the smaller dosage of NA in 0.3 M Na... drinking of > 1 000 ml of water during the second half of the ... riod. ... consumed during any of the other three kinds of infusion

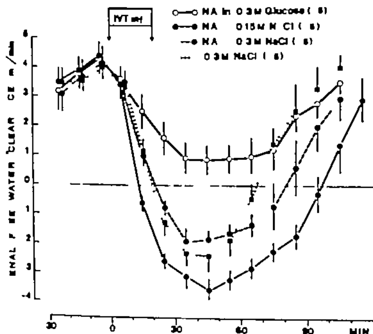


Fig. 1. Cerebral interaction between sodium and noradrenaline (NA) in inhibiting the water diuresis of two ... goats. Dosage of NA 30 pmol/kg min⁻¹. IVT inf = infusion into the third cerebral ventricle at ... µl min. Vertical bars: S.E.

Antidiuresis. As shown in Fig. 1 the most pronounced antidiuretic effect was obtained in response to the NA/0.3 M NaCl infusions which induced negative renal free water clearance (C_{H_2O}) for about 90 min. In contrast, the C_{H_2O} generally remained positive after the NA/glucose infusions. Intermediary antidiuretic effects were obtained in response to the infusions of NA in isotonic saline and of merely 0.3 M NaCl solution.

Renal Na^+ and K^+ excretion. The infusions of NA/0.3 M NaCl induced a pronounced natriuresis culminating 20 to 40 min after the infusion. The peak increase after the larger dosage of NA was 370 ± 50 µmol/min (−210%) with a simultaneous increase in renal K excretion of about 50%. The saluretic effect of the infusions of merely 0.3 M NaCl was roughly half that of the combined infusions (peak increases: Na 110%, K 30%). The rise in renal Na excretion induced by the infusions of NA in isotonic NaCl was insignificant (about 40%), and the NA/glucose infusions, if anything, temporarily reduced renal salt excretion.

Discussion. The integrated results of studies performed predominantly in the goat indicate that juxtaventricular sodium sensitive receptors play an essential role in the cerebral control of fluid balance, and suggest that Na-transporting enzyme activity may be involved in the receptor excitation process (cf. Andersson 1977). The present study demonstrates that small amounts of NA administered into the third ventricle interact with cerebrospinal fluid (CSF) Na in eliciting thirst, apparent ADH-release, and natriuresis. An identical interaction with ... has previously been shown as regards angiotensin II, which has lead to the sugges-

tion that angiotensin may elicit thirst and ADH release by affecting juxtaventricular Na transport in a manner which stimulates sodium sensitive receptors (Andersson 1971). A similar mode of action appears possible also for NA. It would even become likely if adrenaline was found to interact with CSF Na in the same manner since both catecholamines have been shown to activate cerebral Na -K ATPase (Desaiah and Ho 1977).

An approximate estimation based upon figures for CSF bulk flow in the goat (Pappenheimer *et al* 1962) indicates that the lower dosage of NA used might have elevated the NA concentration in the third ventricle to 10^{-4} M This level is too high to allow any speculation about eventual physiological importance of cerebral sodium-noradrenaline interaction in the control of fluid balance However studies in progress will reveal whether the interaction can be demonstrated also with considerably smaller amounts of NA

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From the University Laboratory of Physiology Oxford, England

Short-latency ventilatory responses to sudden withdrawal of hypoxia at normal and raised body temperature in man

By

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Abstract

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...steady isopnoeic conditions (V_E = 40 l/min) were achieved by the inhalation of asphyxial gas mixtures (P_{A,O_2} 60 torr, P_{A,CO_2} 40–45 torr) in normothermia and after ... rise in rectal temperature of ...°C had been induced by ... heated flying suit. Arterial chemoreceptor drive was transiently reduced by ... or isocapnic removal of hypoxia (type (1) tests, two breaths of CO_2 in O_2) or simultaneous withdrawal of hypercapnia and hypoxia (type (2) tests: two breaths of O_2). 8–13 tests of each type were performed ... temperature conditions in 6 expts. on 4 healthy human subjects. Expired volume, total breath duration and inspiratory time were recorded, and minute ventilation and expiratory time subsequently computed breath by breath. In hyperthermia the steady-state ventilation of 40 l/min (at ... relatively higher respiratory frequency and correspondingly lower tidal volume) was achieved at ... P ... CO_2 ... which was 5 torr lower than ... normothermia. Ventilation decreased significantly in all tests. Tested with ... 3-way analysis of variance ... significant differences between the ventilatory responses at the two temperature conditions, and between the two test types were found. The rate of change of ventilation was greater in hyperthermia than in normothermia, and also greater in type (2) tests than in type (1) tests. Since isopnoeic conditions existed prior to the tests, this implies that the arterial chemoreceptor contribution to the total ventilatory drive is increased in hyperthermia. In type (2) tests ... significant lengthening of expiratory time was observed in the ... first breath. This finding confirms the effect in man of changes in airway P_{CO_2} on lung stretch receptor discharge.

...has long been recognized that a rise in body temperature in man is associated with an increased ventilation (Haldane 1905, Cunningham and O'Riordan 1957). Previous studies of the steady state ventilation in man have indicated that this is due to effects on both central regulatory mechanisms and arterial chemoreceptors (Vejby-Christensen and Strange Petersen 1973 a, Strange Petersen and Vejby-Christensen 1977).

It is possible to study the contribution of the arterial chemoreceptors in man directly by ...namic approaches. Inhalation of an asphyxial gas mixture is associated with strong stimulation of the arterial chemoreceptors. As shown by Bouverot *et al.* (1965) and exploited

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in man by Miller *et al* (1974) it is possible to reduce chemoreceptor activity (perhaps to silence them) by transient withdrawal of either CO_2, hypoxia, or both simultaneously.

The present experiments were designed to provide further information about the role played by the arterial chemoreceptors in the control of breathing during hyperthermia in man by studying the dynamic, short latency ventilatory responses to changes in alveolar gas tensions. Transient relief of a moderately strong asphyxial stimulation of the arterial chemoreceptors was achieved either by the simultaneous removal of both the hypercapnic and the hypoxic stimuli, or by isocapnic removal of the hypoxic stimulus.

Methods

Four healthy young men (aged 19–22 years) were studied. Two of them were medical students with knowledge of physiology but none of them were informed about the details of the study, only that it concerned the effect of body temperature on breathing during the inhalation of various gas mixtures. The subjects signed a consent form before the first experiment.

Each expt consisted of two identical sessions, one before and one after rectal temperature had been raised by approximately 1.6 C. Two subjects took part in expts., and two in 1 expt. each. The subjects were encouraged to read throughout the experimental periods.

Breathing apparatus

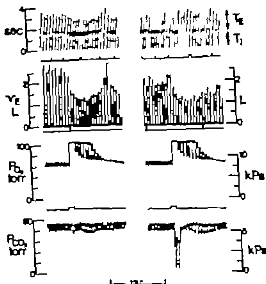
The basic open-circuit employed was like that of Cunningham, Howson and Pearson (1973). The subject breathed warm, humidified air from one of two gas-supply lines through a modified Lloyd valve. Switching between the two lines was performed during expiration by the operation of remotely controlled valves; the subjects were unaware of when switching was performed. The subjects expired through a condenser and a dry gas meter (CD4 Parkinson & Cowan Ltd, London) into a low-inertia wedge spirometer (Oxford Instruments, Oxford) which was emptied during the following inspiration. The dry gas meter provided a quickly available index of mean ventilation which was especially valuable in experiments involving isopnoeic comparisons. Tidal volume (V_T) was determined by integration of the output from the wedge spirometer. Testing with a sinusoidal pump the output of the wedge spirometer was found linear in the range of flow from 5–300 L/min. Inspiratory, expiratory and total breath durations (T_I, T_E and T_T) were determined from the changes in mouth pressure measured by a sensitive manometer (MDC ..., Hilger I.R.D. Ltd, London); they were recorded as a voltage-time ramp re-set at the beginning of inspiration and interrupted briefly at the transition from inspiration to expiration (Fig. 1). End-tidal P_{O_2} and P_{CO_2} were measured with a mass spectrometer (MM 201, V.G. Micromass Ltd, Winsford) sampling at the mouth; calibrations with standard gases previously analyzed on the Lloyd–Haldane apparatus were performed with regular intervals during the experiments. The respiratory variables were recorded by a multi-channel hot-stylus recorder (MR Devices Ltd, Welwyn Garden City, England), and the data were extracted from the records using a P.C.D. trace reader (P.C.D. Ltd, Farnborough, Hants, England).

Heating procedure

The subjects were dressed in a heated flying suit (Junex Electrix Ltd, Glasgow). Innermost they wore light shirt and trousers, and over that a polyethylene suit with constrictions at the wrists and ankles to prevent evaporative heat loss from the trunk and extremities. Over that was the actual heating suit which had heating elements on the front and back of the trunk and around the extremities. Insoles and gloves with heating elements were used periodically. Outside the heating suit the subjects wore an insulating suit with hood and with muffs at the wrists and ankles. The heating suit was supplied with current from three 12 V batteries through a rheostat and an ammeter. By controlling the current it was possible to induce a rise in body temperature and to maintain the temperature within narrow limits of the desired level during the hyperthermic part of the experiments. The heating-up period lasted about 1 h, and the mean rectal temperature rise in the expts. was 1.66°C (Table I). During the warming-up period the subjects were given salted fruit juice (400–600 ml) to replace their losses through sweat production.

Temperature measurements

Rectal temperature was measured with a thermocouple placed at a depth of cm from the anus and ... (...ers, Ellab, Copenhagen).



[illegible] wo specimens of experimental record [illegible] left an example of type (1) test [illegible] removal of hypoxia), on the right [illegible] 2) test (simultaneous removal of both [illegible] and hypercapnia). From above down-[illegible] breath duration (s) split into T_I (below [illegible] line) and T_E (above break); marker [illegible] meter, signals indicating the passage [illegible] expiratory volume v_E (L), the increasing $V_{T,E}$, P_{O_2} and P_{CO_2} at the mouth [illegible] by event marker showing the three [illegible] hich test gas was inhaled. The P_{O_2} [illegible] tro, traces lag behind the others by [illegible] ort delay of about 1.5 [illegible] Note that the [illegible] d P_{O_2} does not return to the steady [illegible] level immediately after the test. This is [illegible] the fact that the space between the [illegible] alve and the solenoid valve in the test [illegible] pply line after its closure is poorly [illegible] ted space (cf. Cunningham et al. 1973 [illegible])

[illegible] mental procedure

[illegible] asic procedure employed in the present study was to replace [illegible] steady asphyxial mixture with oxygen [illegible] (O_2 in oxygen) for two breaths. A moderately strong arterial chemoreceptor driv[illegible] (P_{A,O_2} 60 torr (8.0 [illegible] P_{A,CO_2} 40–45 torr (5.3–6.0 kPa)) was induced by giving the subjects [illegible] gas mixture with [illegible] P_{O_2} about [illegible] (9.9 kPa) and [illegible] P_{CO_2} of 32–38 torr (4.2–5.1 kPa). W[illegible] attempted to achieve isopnoic conditions ([illegible] in ventilation of approximately 40 l/min) in the steady state at each of the two temperature levels by [illegible] tment of the inspired CO_2 concentration. This gas mixture was breathed for at least 15 min before [illegible] were performed [illegible] ith intervals of at least 3 min between each. Two different types of test [illegible] were per[illegible]: *Test type (1)*, isocapnic removal of hypoxia, the test gas being oxygen [illegible] ith CO_2 added so as to [illegible] tain an unchanged P_{A,CO_2} during and after the test. *Test type (2)*, simultaneous removal of hypoxia [illegible] hypercapnia, the test gas being oxygen. In both test types the test gas was given for tw[illegible] breaths (breath [illegible] d breath 1) after [illegible] hich the subjects were switched back to the steady-state mixture with smaller modi-[illegible] tions of the CO_2 concentration in order to maintain P_{A,CO_2} at the same level as before the test. P_{A,O_2}

[illegible] LE 1 For each of the two test types at the two temperature conditions studied in each expt. are shown [illegible] rectal temperature (°C), mean pre-test P_{A,CO_2} (torr), maximal P_{A,CO_2} change with test, and mean pre-test breath duration (T_T, sec) and ventilation (V_E, L/min)

	Temp	Test type (1)				Test type (2)			
		P_{A,CO_2}	$\Delta P_{A,CO_2}$	T	V	P_{A,CO_2}	Δ_{A,CO_2}	T	V
AJ2	36 30	43 4	0 8	3 06	37.3	43 8	13 9	3 03	36.3
	37 70	42 2	0 2	2 41	45.9	43 0	13.2	2.45	45.5
LL	36 45	42 6	0 3	1 76	40 8	43 1	14 6	1 82	40 0
23	37 65	39 1	0 6	1.32	42.6	39 4	11.2	1.38	41.6
	36 15	44 5	0 3	1 90	37.3	44 4	16 8	2.00	37 8
	37 80	39 8	0 6	1.61	39 7	40 0	12 3	1.63	39 6
17	36 20	43 2	0 3	3 31	38 2	43.1	12.1	3 32	38 1
18	38 10	37 7	0 9	2.90	37 0	37 6	7 9	2.78	36 0
	36.10	46.2	0 6	3 49	39 3	46.1	14 4	3 32	38.5
	38 40	37.8	0 7	2.56	37 9	38.2	7 4	2 83	36 4
04	37 15	46.3	1 6	2.31	42.1	46 5	10.9	2.39	40 6
	38 65	39 4	0 9	2.20	40 8	39 4	7 1	2 39	40.2

TABLE II Three-way analysis of variance of the effects of *A. temperature B. test type* and *C* breath (0–4) on minute ventilation calculated breath by breath.

	434 12	462.2	462.3	411 7	411 8	[illegible]
A	+ + +	+ +	NS	+ + +	+ +	
B	+ +	+ +	+ + +	+ + +	+ +	
C	+ + +	+ + +	+ + +	+ + +	+ + +	[illegible]
A B	NS	+ +	+	NS	NS	
A C	NS	NS	+ +	NS	NS	[illegible]
B C	NS	NS	+ +	NS	NS	[illegible]
A B C	NS	NS	NS	NS	NS	

+ + + = p < 0 001 + + = p < 0.01 + = p < 0.05 NS = p > 0 05

increased in both test types to more than 200 torr (27 0 kPa) in breath 0 P_{A,CO_2} fell by 10–14 torr (1. kPa) in type (2) tests (*cf* Table I). The sequence of the tests were randomized, each expt. compri to thirteen successful tests of each type in each temperature condition.

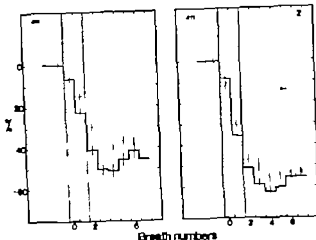
Results

Fig. 1 shows two segments of an original record (expt. 411 7), on the left a type (1) tes on the right a type (2) test It shows the steep rise and subsequent slow return of P_A and for $P_{A\ CO_2}$ the approximate constancy in type (1) and the drop in type (2). The assoc changes in expired volume (v_E) are obvious. V_T, T_I and T_E were measured for breaths −1 0 1 2, 3 4 5 6 and 7 and expired minute ventilation (V_E) was subsequently calcul for each of these breaths.

In the statistical treatment of the data a 3-way analysis of variance (Sokal and R 1969) was performed The number of tests in each experiment was equalized (8-1 exclusion of tests using random numbers The analysis was carried out on the abs values of minute ventilation of breaths 0, 1 2, 3 and 4 breaths 5 6 and 7 were not incl in this analysis since only ventilatory changes occurring within 10–12 sec of a test (Bou *et al* 1965 Miller *et al* 1974) are indicative of relief of arterial chemoreceptor drive. In three way analysis of variance the effects of body temperature, test type and breath nu were tested The results are shown in Table II Significant effects related to all three fa were found in all experiments with the exception of temperature in experiment 462.3 effect of breath number merely reflects the temporal pattern of the response (Fig. 1 an In all but one expt. a rise in body temperature was thus found to cause a significantly ferent ventilatory response to tests of both types, and in all expts. the responses to the test types were different at both levels of body temperature.

Fig. 2 shows the average changes in ventilation as a function of breath number d and after both test types in both temperature conditions in expt. 411 7 which largely re the general findings. It is seen how $\dot{V}_E$ decreased following the tests, and how for bot types the responses were larger in the hyperthermic than in the normothermic cond Comparing the two test types further shows that the responses in both temperature c tions were larger following tests of type (2) than of type (1).

As stated in methods it was intended that the steady-state ventilation in both tem ture conditions was the same, 40 l/min. This was achie



Mean percentage changes in ventilation during and ... (1) tests (left) and type (right) at normal (broken ... elevated body tempera... line) in expt 411 7 The ... area corresponds to the ... (0 and 1) during which ... gas ... inhaled.

... I) in all but 1 expt. (434 12), where the ventilation in hyperthermia was some 8–9 ... higher than in normothermia. However an analysis of variance performed on the ... of changes in ventilation from the control values (the means for breaths 2 and −1) ...led also in this expt. significant effects of both temperature and test type on the res...

...n attempt to quantitate the differences due to test type and to body temperature the ...sion slopes of the absolute values of minute ventilation on breaths numbers 0 to 4 ... calculated (Table III). The slope thus calculated is an index of the mean fall in ventila... per breath during and after tests. It was numerically greater in hyperthermia than in ...nothermia in 10 out of 12 cases (the exceptions being 462.2 in type (1) tests and 411.8 ...type (2) tests). The mean difference in slope was 1 l/min/breath. Comparison of the two ...types showed larger slope values in type (2) tests in 9 out of 12 cases (the exceptions ...g 462.2 in normothermia and 411.8 and 440.4 in hyperthermia). The mean difference ...lope was 0.5 l/min/breath

...he transient ventilatory responses were largely due to decreases in tidal volume respira...y frequency usually remained constant although in some tests either a small decrease

...LE III Regression analysis of ventilation (calculated for individual breaths) against breath numbers 0–4 V ... b breath number ... Only slope values (b, l min⁻¹ per breath) are shown.

...pt.	Normothermia Type (1)	Normothermia Type (2)	Hyperthermia Type (1)	Hyperthermia Type (2)
	b	b	b	b
4 12	0 8	1 7	..7	3 7
2.2	2.3	1 6	−1 7	−2.7
...3	1 9	2.7	2 8	3 4
1 7	3 6	−4 4	4 4	−4 9
11 8	3 4	−4 9	4.5	4 4
[illegible]	3.1	−4 3	6 3	5 1

(= lengthening of T_T *cf* Fig. 1 right) or less commonly a small increase (shorter T_T *cf* Fig. 1 left) was observed A detailed analysis of the respiratory pattern in the state and during the transient responses revealed results which are largely confirm previous findings these results will therefore not be presented here.

Discussion

At normal body temperature the results obtained in the present study regarding time c and magnitude of the ventilatory responses to tests were largely comparable to prev published data from this laboratory (Miller *et al* 1974 Cunningham, Drysdale and 1976) This agreement like arguments presented elsewhere (Strange Petersen and Christensen 1977) would argue against any unspecific effects associated with the suit or apprehension in anticipation of the "warm" session affecting the responses

For both test types, however the analysis of variance showed significantly di responses following the induction of a 1 6 C rise in rectal temperature. The quan estimate obtained by the regression analysis (Table III) revealed larger ventilatory res in hyperthermia than in normothermia in 5 out of the 6 expts.

It should be emphasized that the steady-state $P_{A\ CO_2}$ was different in the two tempe conditions In normothermia the ventilation of 40 l/min was achieved at a P_{A,O_2} of (8 0 kPa) and a $P_{A\ CO_2}$ of 44.8 torr (6 0 kPa) in hyperthermia at a P_{A,O_2} of 60 torr (and a P_{A,CO_2} of 39 4 torr (5 2 kPa) (mean values, *cf* Table I). For the overall ch control of ventilation it was thus found that a rise in body temperature of 1.6°C h same effect as an increase of $P_{A\ CO_2}$ of *ca* 5 torr (0 7 kPa) Similarly in tests of type ratio $\Delta\dot{V}_E/\Delta P_{A\ CO_2}$ was larger in hyperthermia than in normothermia.

Inspection of Fig. 2 shows decreases in ventilation in breath 0: such early change most commonly observed in type (2) tests. They occurred too early to be arterial c receptor responses and can probably partly be explained by the increase in viscosi density in the high O test breaths. In type (2) tests a lengthening of the expiratory (T_E) was observed in breath 0 this confirms findings published and discussed full where (Cunningham *et al* 1977) and provides further evidence that interaction b stretch receptor activity and airway P_{CO_2} (Mustafa and Purves 1973, Bradley Nobl Trenchard 1976) is present and active in man. We found the lengthening of T_E be normothermia (80 ms ±22, n = 63) than in hyperthermia (50 ms ±30, n = 68) this migh be due to a greater change in $P_{A\ CO_2}$ in normothermia than in hyperthermia (about versus about 10 torr) Consequently these findings do not permit any conclusions whether the stretch receptor—P_{CO_2} interaction is modified by changes in body tempe (see below). The lengthening of T_E (and T_T) contributed to the observed reductio ventilation in breath 0 in type ( ) tests In type (1) tests, on the other hand only a and insignificant shortening of T_E was found in breath 0 in either temperature condi

In both normo- and hyperthermia the ventilatory responses to tests of type (2 consistently larger than those to type (1) a finding which agrees with that of a pr study on man (Miller *et al* 1974) It seems likely that this difference may in part be the dynamic response properties of the chemoreceptors. Th on th

-oakey and Torrance (1971) observed no transient over or undershoot in the carotid -nerve activity upon induction or relief of hypoxia (*cf* our test type (1)) whereas re l of hypercapnia alone (*cf* our test type (2)) caused a transient decrease to zero activity

-e main finding of the present study was that the ventilatory response to tests of either as larger in hyperthermia than in normothermia, significantly so in 5 of the 6 expts. hort latency reduction in ventilation following an O_2-test is due to a fall in the input the arterial chemoreceptors to the respiratory centre. Since the present comparison based upon virtually isopnoeic conditions in the steady-states between tests, the larger onses found in hyperthermia indicate that the contribution to ventilation attributable e arterial chemoreceptors was also relatively increased in this state. Whether or not al interaction between peripheral and central chemoreceptor input takes place, this ng implies that that part of the total ventilatory drive which depends upon peripheral noreceptor activity is increased in hyperthermia. These results thus confirm our previous dy-state findings of an enhanced ventilatory response to hypoxia in hyperthermia (Vejby-istensen and Strange Petersen 1973 a, Strange Petersen and Vejby-Christensen 1977) are consonant with the contention that this is due to an increased arterial chemoreceptor ity Evidence from animal work that this is the case has most recently been presented McQueen and Eyzaguirre (1974).

he hyperventilation associated with rise of body temperature in the absence of hypoxia ldane 1905, Cunningham and O'Riordan 1957 Strange Petersen and Vejby-Christensen 77) where peripheral chemoreceptor contribution is believed to be small, suggests that perthermia also affects intracranial chemosensitivity

A simple, unspecific sensitization of "respiratory centre" neurons caused by a rise in perature, on the other hand, could hardly explain why the increase in ventilatory response hypoxia produced by a certain elevation of the body temperature is relatively larger than e increase in ventilation produced by the same temperature elevation (when air is inspired). ur previous finding, that the magnitude of the fast ventilatory response at the onset of ercise was unaffected by hyperthermia (Vejby-Christensen and Strange Petersen 1973 b) so argues against such unspecific sensitization. More direct evidence against this idea, ually has been presented by Chai, M and Brobeck (1965) and by Tabatabai (1972) who ound decreases in both tidal volume and respiratory frequency in response to localized eating of areas in the medulla corresponding to the respiratory centres.

Besides affecting the intracranial and peripheral chemosensitivity rise in body tempera-ure has also a third, direct effect on the pattern of breathing, which has been fully confirmed y all steady-state measurements of ventilation, breath duration and tidal volume in the resent study (Table 1). It affects the ponto-medullary mechanisms responsible for the etting of respiratory rhythm so that a given ventilation is achieved at a relatively higher requency and a correspondingly lower tidal volume (Hey *et al.* 1966, Euler Herrero and Wexler 1970, Euler and Trippenbach 1976, Strange Petersen and Vejby-Christensen 1977).

Some authors (Schoener and Frankel 1972, Grunstein *et al.* 1973), finally have reported evidence of a fourth effect, a rise in temperature causing an increase in the vagal feed-back from stretch receptors in the lungs. As briefly indicated above our present data do not provide any evidence as to the importance of this in man.

The work was supported by the Danish and British Medical Research Councils. J. I. J. ... a Florey Scholarship at the Queen's College, Oxford. Technical, clerical and secretarial ... provided by Messrs E. Aldsworth, D. V. Goff, M. G. Howson, T. J. Meadows and Mrs L. ...

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From the Department of Pharmacology University of Copenhagen, Denmark

The mechanism of histamine release induced by the ionophore X537A from isolated rat mast cells

I. Significance of Monovalent Cations, Calcium, Metabolic Energy and Temperature

By

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Abstract

...ierczak, W. S. A. Patkar and B. Diamant. *The mechanism of histamine release induced by the ionophore X537A from isolated rat mast cells. I. Significance of monovalent cations, calcium, metabolic energy and temperature.* Acta physiol. scand. 1978. 102. 265–273

...ine release induced by X537A from isolated rat mast cells was dependent on the presence of sodium ... but not on calcium in the incubation medium. The rate of release was faster in potassium ... sodium, but the final release was the same after prolonged incubation. Extracellular calcium as ... metabolic inhibitors depressed the rate of release induced by X537A. Pretreatment of mast cells ... EDTA or the ionophore A23187 in the absence of extracellular calcium did not influence sub... release induced by X537A. The findings of release at 45°C and lack of release at 0°C distinguish ... mechanism of action of X537A from calcium- and energy-dependent releasing agents like compound ... and A23187 on the one hand and the simple lytic action of decylamine on the other.

...phoreous antibiotics A23187 (Eli Lilly & Co.) and X537A (Lasalocid) (Hoffmann-La ...he) are able to transport cations across biological membranes (Caswell and Pressman ..., Reed and Lardy 1972 a and b, Pressman 1973). They have been used in recent years ...tudy the involvement of calcium and other ions in various secretory processes. A23187 ... more specific carrier of calcium than X537A, which in addition has been shown to ...nsport monovalent cations as well as some primary amines (Pressman 1973). Further...re, X537A has been shown to exert a greater depolarizing action than A23187 on frog ...letal muscle fibers (Cochrane and Douglas 1975). Therefore, some of the functional ...ponses observed for X537A might be ascribed to other effects than to an enhanced ...nsport of calcium. The dependence of X537A on calcium for histamine release from ...lated rat mast cells has been a subject of conflicting opinions (Foreman *et al.* 1973, Cochrane and Douglas 1974, Kagayama and Douglas 1974). The present investigation was performed in order to clarify the mechanism by which X537A elicits the release of histamine from isolated rat mast cells.

Methods and materials

All solutions contained 1 mg/ml of dialysed bo ine serum albumin and, unless otherwise st glucose The following solutions were used in the present investigation.

1) *Sorensen-A* consisted of 144 mM NaCl, .7 mM KCl buffered with Sorensen phosph mM pH 7 0 at room temperature, 2) *Tri A* with similar composition as Sorensen-A, ex phosphat buffer was substituted with 10 mM Tris-HCl, pH 7.2 at room temperature contained 300 mM glucose buffered with 10 mM Tris-HCl, pH 7.., at room temperature 5) *Tris-pot sium* and 6) *Tris-calcium* which were simila to Tris-glucose, except that glucose by increasing concentrations of NaCl (10–150 mM), KCl (10–150 mM) or $CaCl_2$ (10–150 m

Isolation of rat mast cells

Mast cells from the abdominal and thoracic ca ities of male and female Wistar rats (exp were harvested and isolated by centrifugation in a Ficoll density gradient (Thon and Uvn isolation of the mast cells they were washed 3 times by centrifugation at 200 *g* for 5 min, wi or Tris-A s washing solution. I some expts, the last w sh wa performed in Tris-glucose

Incubation procedure

In all expts. triplicate samples for each experimental condition were carried out and the me used for calculations. The spontaneous release of histamine observed in the absence of re has been deducted from all values presented except when otherwise stated. The values of th release in different incubation media re gi en n the text.

The cells were incubated either d luted (5 10³ cell in 2 ml) or concentrated (2.5 10⁵ c in a relevant incubation solution. The reaction was usually started by the add tion of hista agents. After ppropriat incubation time the reaction was stopped by placing the incubatio for the diluted cells or by transferring 70 µl (approximately 5 000 cells) to 2 ml ice-cold usually was of the same composition s used during incubation.

Hi tamine determination

Following incubation the cells were centrif ged at 350 *g* for 10 min at 4°C. The supernatan l to new tubes and ml 0 08 N HCl was added to the cell sediments t lyse the cells. By add acid the pH of the supernatants was adjusted to the same value as that of the solutions of hi mine w s determined fluorometrically (Shore *et al* 1959), omitting the extraction procedu and U nås 1972, Diamant and Patkar 1975). Histamine release was calculated as a percent histamine content of each cell sample. All lues are expressed as mean ± S.E.

Chemicals

W are greatly indebted to H ffmann–La Roche (Basel Switzerland) for the generous gi to Eli Lilly & Co (Indianapolis, USA) for A23187 as w ll as to AB Leo (Helsingborg, Sw pound 48/80

X537A was dissolved i dimethylsulfoxide (DMSO) t btain final concentration of was dissolved accord g to Prince *et al* (1973) to give stock solution of 10^{-4} M Both stock kept at −20°C. All further dil tions were made i ppropriate ncubation media. The final of acetone, ethanol and DMSO in the incubation mi ture were n all cases too low to influ release Ficoll was purchased from AB Pharmacia (Uppsala, Sweden), bovine serum albumi (AM), and -deoxyglucose (2 DG) from Sigma Chemical Corp. (St Louis, USA), and o-p and decylamine from Fluka AG (Buchs, Switzerland).

Results

The influence of calcium on X537A-induced release

When cells were suspended in Sorensen-Amedium the release duced by X centrations from 5 10^{-6} to 5 10 M gradually increas 80

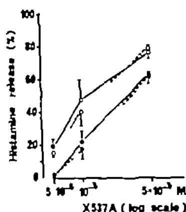


Fig. 1

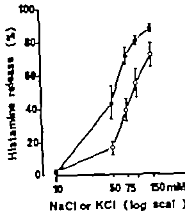


Fig. 2

The inhibitory action of calcium (1 mM) on histamine release induced by X537A. Mast cells were [illegible] to increasing concentrations of X537A in the presence (filled symbols) or absence (open symbols) [illegible] calcium. The cells were either preincubated with calcium for 10 min and the reaction started [illegible] X537A (dotted lines), or X537A was preincubated together with calcium and the reaction was started [illegible] cells (continuous lines). Incubation time: 10 min. Each point represents mean ± S.E. of 3–4 expts.

2. The influence of increasing concentrations of NaCl (open symbols) or KCl (filled symbols) on [illegible] release induced by X537A (10^{-4} M). After isolation mast cells were washed twice with Tris-A [illegible] with Tris-glucose before exposure to X537A in Tris-sodium or Tris-potassium. Mean ± S.E. of [illegible]

[illegible]mine content (Fig. 1). At each point investigated, the presence of 1 mM calcium de[illegible]sed the release, irrespective of whether the cells were preincubated with calcium for 10 [illegible] prior to the addition of X537A or whether the reaction was started by the addition of [illegible] to the incubation medium containing X537A and calcium. It was further observed that [illegible] suboptimal release induced by 10^{-4} M of X537A after 10 min gradually increased during [illegible]longed incubation, also in the presence of calcium, indicating that calcium decreased the [illegible] of the release process (data not shown).

[illegible] 6 expts. performed in Tris-glucose, X537A was found to be ineffective to release hista[illegible] above the spontaneous release after 10 min of incubation. In the same expts., when [illegible]cose was gradually substituted with increasing concentrations of calcium up to 110 mM [illegible] no histamine was released by X537A (10^{-4} M). However, in Tris-calcium (110 mM) [illegible] as in Tris-glucose the spontaneous release was elevated to 29.3 ± 2.8% and 17.1 ± [illegible]%, respectively, as compared with 6.5 + 0.7% in Tris-sodium (150 mM).

When mast cells were preincubated for 2 h at 37°C in Sørensen-A before exposure to [illegible] (1 mM) and compound 48/80 or to compound 48/80 alone, the secretory response [illegible] decreased. When EDTA (1 mM) was present during the preincubation period a [illegible] decrease of the release was found after challenge with 48/80 alone, but not after [illegible] addition of compound 48/80 and calcium (Table I). In contrast, the response [illegible] mast cells to X537A in the absence of calcium was unaffected by pretreatment of the cells with EDTA (1 mM) for 2 h. Furthermore, pretreatment of mast cells in Sørensen-A with the [illegible] A-3187 (10^{-4} M), which completely abolished histamine release induced by

TABLE I The influence of EDTA (1 mM) on histamine release induced by compound 48/80 and X537A (10^{-4} M)

Preincubation	Histamine release (%)			
	48/80 (n = 7)		P_{diff}	X537A (n = 3)
	− Ca	+ Ca		Ca
3 min	43 4 ± 5 7	63 8 ± 4.8	< 0.001	56 0 ± 11 5
2 hrs	30 4 ± 3 0	46.6 ± 4 4	< 0 01	48 2 + 6 6
2 hrs with EDTA (1 mM)	16 4 ± 3 4	49 4 ± 3.3	< 0.001	41 2 + 1 6

Following preincubation in Sorensen-A at 37°C as described in the table the cells were diluted into Sorensen-A containing 48/80 or X537A and eventually calcium (1 mM). Incubation was then for 10 min. P_{diff} 48/80 3 min 2 h (− Ca) < 0.05 3 min 2 h (+ Ca) < 0 01 2 h EDTA (+ Ca)-2 h (− Ca) < 0.001

compound 48/80 did not influence histamine release induced by X537A or de (Table II)

The influence of monovalent cations on histamine release induced by X537A

When mast cells were incubated in Tris-sodium or Tris-potassium containing in concentrations of the monovalent cations and exposed to X537A (10^{-4} M) for histamine release was evident in the presence of 50 mM of either ion. The release by increasing their concentration and at each concentration the release observed presence of potassium was markedly higher than that obtained with sodium (Fig 2 spondingly incubations with X537A (7.5 10^{-4} M) for various periods in Tris-p (150 mM) and Tris-sodium (150 mM) showed that the rate of release was faster in th medium, but the total release after prolonged incubation was the same (80 %) in bo (Fig. 3).

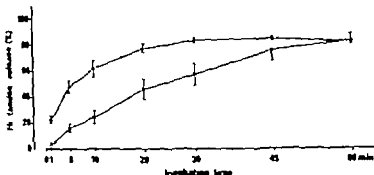
The influence of temperature

When mast cells were preincubated in Sorensen A for 10 min at different temperat fore the addition of compound 48/80 (0 6 µg/ml), X537A (10^{-4} M) or A23187 (together with calcium (1 mM) none of the agents released histamine at 0°C (Fi this respect they differed from decylamine (30 µg/ml), which in 4 expts. released h equally well at 0°C as at 37°C (data not shown) The release induced by X537A I increased with the incubation temperature and was more pronounced at 45°C

TABLE II The influence of pretreatment of mast cells with the ionophore A23187 on subsequent release induced by compound 48/80 (0.6 µg/ml), X537A (10^{-4} M) and decylamine (30,

Releasers	Histamine release (%)	
	Control cells	Ionophore-treated cells
48/80	43.9 ± 1.3	0 6 ± 0.4
X537A	65.9 ± 11 7	66.3 ± 10.3
Decylamine	79.5 ± 12.4	80.3 ± 12.4

Mast cells were preincubated with 10^{-6} M of A23187 for 3 min (Diamant and then incubated for 10 min in the presence of releasing agents. Mean

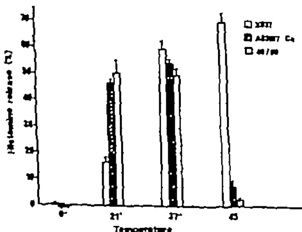


The time course of histamine release induced by X537A in Tris-sodium (150 mM) (open symbols) -potassium (150 mM) (filled symbols). After isolation mast cells were washed twice in Tris-A and Tris-glucose and then incubated concentrated in 100 µl Tris-sodium or Tris-potassium containing (7.5 · 10^{-6} M) for increasing lengths of time. The reaction was stopped by transferring 20 µl of ... to 2 ml ice-cold Tris-glucose. Each point represents mean ± S.E. of 4–6 expts.

A23187 and compound 48/80 effectively released histamine at both 21°C and 37°C, virtually no release was induced by these agents from cells incubated at 45°C. After 10 ... of incubation, X537A released less histamine at 21°C than the other two releasing ...ts. However by prolonging the incubation time at 21°C, the release induced by X537A ...ually increased, so that after 90 min the release amounted to the same values as observed ...°C (97% of the total histamine content, data not shown).

Influence of metabolic energy

...ed mast cells were incubated in Sørensen-A together with 10^{-6} M of Antimycin (an ...bitor of oxidative phosphorylation) for 10 min prior to the addition of various concentra-



...g. 4. The influence of temperature on histamine release induced by compound 48/80, X537A or A23187 together with calcium. Mast cells were isolated and preincubated in 100 µl Sørensen-A for 10 min at each temperature before the addition of releasing agents. The incubation was continued for 10 min at the respective temperature, except at 0°C, which was prolonged to 20 min. The reaction was stopped by dilution ... 100 µl to 2 ml ice-cold Sørensen-A. Mean ± S.E. of 3 expts.

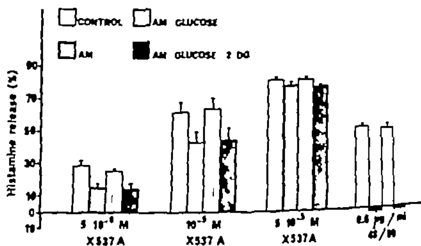


Fig. 5 The influence of antimycin, glucose and 2-deoxyglucose on histamine release
and compound 48/80. After addition of X537A or compound 48/80 incubation
Mean ± S.E. of 4 expts.

tions of X537A, it was noted that Antimycin induced a slight, but significant
concentrations of X537A which caused submaximal release (Fig. 5) This
counteracted by the simultaneous presence of glucose (2 mM) in the medium. The
ing effect of glucose was, in turn abolished by the simultaneous presence of
deoxyglucose (an inhibitor of glucose utilization) At higher concentrations of
($5 \quad 10^{-4}$ M) no influence of these metabolic inhibitors was observed. For compar
histamine release induced by compound 48/80 was included, showing a complete
by Antimycin, which was reversed by the presence of glucose This effect of
completely counteracted by the presence of 10 mM 2-deoxyglucose. The inhibitory
of Antimycin together with 2-deoxyglucose on histamine release induced by X537A
10^{-4} M) from rat mast cells incubated in Sorensen-A was overcome by prolonged
of the cells with the releasing agent (4 expts, data not shown) The inhibitory action
metabolic inhibitors was notable at 10 and 20 min of exposure of the cells to X537
was absent at 30 and 45 min of exposure, in which cases the histamine release
70 or more. Thus, these agents decreased the rate of release induced by X537A
influencing the total release

Discussion

The present results agree with those of Foreman *et al* (1973) who found that
extracellular calcium did not inhibit histamine release induced by X537A. In fact,
hands extracellular calcium was found to depress the secretory action of X537A.
more, in the absence of extracellular calcium pretreatment of the cells with either
or A23187 which effectively diminished the response of the cells to compound 48/80
las and Ueda 1973 Diamant and Patkar 1975) did not, in our hands, influence the
action of X537A These results differ from those of Douglas and coworkers (Cochr
Douglas 1974 Kagayama and Douglas 1974) who found that EDTA-treated cells
respond to X537A unless calcium was added to the cells. T differen

EDTA-treated cells is at present not clear. Our finding that there was no influence EDTA or A23187 on histamine release by X537A does not favour the opinion mechanism of release depends on intracellular calcium. In our hands the presence calcium depressed the secretory response of mast cells to X537A. The finding that ct was evident when the cells were added to an incubation medium containing and calcium as well as when they were preincubated with calcium before the addition A indicates that inactivation of X537A by binding to calcium is not a likely explana- is further supported by the finding (unpublished observations) that barium, as a much greater affinity to X537A than calcium (Pressman 1973), was ineffective on the release induced by X537A. Since calcium was found to depress the rate of release by X537A, a more likely explanation for the inhibitory action of extra calcium might be a stabilizing effect on the plasma membrane of mast cells, which interfere with either uptake of X537A into the cells or with transport of monovalent across the plasma membrane. An inhibitory effect of calcium on the secretory action 7A has also been reported for insulin secretion from pancreatic β-cells (Hellman

present results indicate that monovalent cations are inherent in the mechanism by X537A releases histamine from rat mast cells. In this respect our results agree with ations concerning the release of vasopressin induced by X537A from neurohypophysis non et al. 1976). Monovalent cations are known to exchange for histamine bound to ular matrix (Uvnäs et al. 1970). Since they are carried by X537A over membranes man 1973), we suggest that they reach and release histamine from intracellular granules the requirement of a calcium- and energy-dependent exocytotic process to establish ions for exchange. The finding that the rate of histamine release was faster in Tris- sium (150 mM) as compared with Tris-sodium (150 mM) is in agreement with observa- concerning the influence of these ions on X537A-induced release of serotonin from platelets (Murer et al. 1976). This effect could be ascribed to a depolarization of the membrane of intact rat mast cells by high potassium (Gusein 1973), which could enhanced by X537A, which by itself is known as a depolarizing agent (Cochrane and ... 1975). An additional possibility might be that potassium is more efficiently trans- ported across membranes by X537A than sodium. An action of high potassium by itself on cells is suggested from the findings that the spontaneous release was higher in Tris- sium (150 mM) than in Tris-sodium (150 mM) and amounted to 21.1 ± 3.2% and 9.7 ... respectively of the total histamine content after 10 min of incubation (mean ± of 6 expts.)

Although the rate of release of histamine was found to be dependent on the concentration 537A, it was generally slower than those observed with several agents which depend on ions or metabolic energy for their secretory effects (compound 48/80, antigen, and 187) (Bloom et al. 1967. Diamant et al. 1974. Diamant and Patkar 1975). The release serotonin from blood platelets induced by X537A has also been noted to be slow as com- pared with that induced by other releasing agents (Murer et al. 1976).

The influence of metabolic inhibitors on the secretory action of X537A on mast cells had n studied earlier by Foreman et al. (1973), who found that neither cyanide nor 2-deoxy-

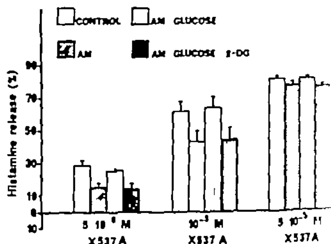


Fig. 5. The influence of antimycin, glucose and 2-deoxyglucose o[illegible] and compound 48/80. After addition of X537A or compound 48/80 Mea[illegible] ± S.E. [illegible] 4 expts.

tions of X537A, it was noted that Antimycin induced a sligh[illegible] concentrations of X537A which caused submaximal releas[illegible] counteracted by the simultaneous presence of glucose (2 mM) [illegible] ing effect of glucose was, in turn, abolished by the simulta[illegible] deoxyglucose (an inhibitor of glucose utilization) At hig[illegible] (5 10⁻ M) no influence of these metabolic inhibitors was ob[illegible] histamine release induced by compound 48/80 was included sh[illegible] by Antimycin, which was reversed by the presence of glucose completely counteracted by the presence of 10 mM 2-deoxyglu[illegible] of Antimycin together with 2-deoxyglucose on histamine release [illegible] 10^{-4} M) from rat mast cells incubated in Sorensen-A was overcome [illegible] of the cells with the releasing agent (4 expts data not shown). Th[illegible] metabolic inhibitors was notable at 10 and 20 min of exposure of t[illegible] was absent at 30 and 45 min of exposure, in which cases the histamin[illegible] 70 or more. Thus, these agents decreased the rate of release induc[illegible] influencing the total release.

Discussion

The present results agree with those of Foreman *et al* (1973), who four[illegible] extracellular calcium did not inhibit histamine release induced by X5[illegible] hands extracellular calcium was found to depress the secretory action of more, in the absence of extracellular calcium, pretreatment of the cells w[illegible] or A23187 which effectively diminished the response of the cells to compo[illegible] las and Ueda 1973 Diamant and Patkar 1975), did not in our hands, influe[illegible] action of X537A These results differ from those of Douglas and coworkers [illegible] Douglas 1974 Kagayama and Douglas 1974), who found that EDTA-treated respond to X537A unless calcium was added to the cells. The reason for the di[illegible]

EDTA-treated cells is at present not clear. Our finding that there was no influence EDTA or A23187 on histamine release by X537A does not favour the opinion mechanism of release depends on intracellular calcium. In our hands the presence calcium depressed the secretory response of mast cells to X537A. The finding that t was evident when the cells were added to an incubation medium containing d calcium as well as when they were preincubated with calcium before the addition indicates that inactivation of X537A by binding to calcium is not a likely explana- s is further supported by the finding (unpublished observations) that barium, s a much greater affinity to X537A than calcium (Pressman 1973), was ineffective the release induced by X537A. Since calcium was found to depress the rate of release by X537A, a more likely explanation for the inhibitory action of extra alcium might be a stabilizing effect on the plasma membrane of mast cells, which terfere with either uptake of X537A into the cells or with transport of monovalent across the plasma membrane. An inhibitory effect of calcium on the secretory action A has also been reported for insulin secretion from pancreatic β-cells (Hellman

resent results indicate that monovalent cations are inherent in the mechanism by X537A releases histamine from rat mast cells. In this respect our results agree with tions concerning the release of vasopressin induced by X537A from neurohypophysis on et al. 1976). Monovalent cations are known to exchange for histamine bound to ular matrix (Uvnäs et al. 1970). Since they are carried by X537A over membranes an 1973), we suggest that they reach and release histamine from intracellular granules t the requirement of a calcium- and energy-dependent exocytotic process to establish ons for exchange. The finding that the rate of histamine release was f ster in Tris- um (150 mM) as compared with Tris-sodium (150 mM) is in agreement with observa concerning the influence of these ions on X537A-induced release of serotonin from platelets (Murer et al. 1976). This effect could be ascribed to a depolarization of the membrane of intact rat mast cells by high potassium (Guscin 1973), which could nted by X537A, which by itself is known as a depolarizing agent (Cochrane and les 1975). An additional possibility might be that potassium is more efficiently trans- across membranes by X537A than sodium. An action of high potassium by itself on cells is suggested from the findings that the spontaneous release was higher in Tris- sium (150 mM) than in Tris-sodium (150 mM) and amounted to 21 1 ± 3.2% and 0 7 % respectively of the total histamine content after 10 min of incubation (mean ± of 6 expts.).

though the rate of release of histamine was found to be dependent on the concentration 537A, it was generally slower than those observed with several agents which depend on ons or metabolic energy for their secretory effects (compound 48/80, antigen, and 187) (Bloom et al. 1967. Diamant et al. 1974. Diamant and Patkar 1975). The release otonin from blood platelets induced by X537A has also been noted to be slow as com- with that induced by other releasing agents (Murer et al. 1976).

he influence of metabolic inhibitors on the secretory action of X537A on mast cells had udied earlier by Foreman et al. (1973) ...

glucose influenced the action. The present investigation has in general confirmed results by using Antimycin and 2-deoxyglucose as metabolic inhibitors, although depression of the rate of release was observed at suboptimal concentrations of X537A indicates that cellular ATP might affect the permeability properties of mast cell membranes by mechanisms which are at present unknown.

An additional finding which distinguishes the mechanism by which histamine is released by X537A from that induced by the ionophore A23187 and compound 48/80 was the observation that at 45 C X537A effectively released histamine, whereas the other two agents failed to do so. The action of X537A could also be distinguished from the simple lytic effect of decylamine since X537A did not release histamine at 0 C, whereas low temperature did not abolish the releasing effect of decylamine.

It is concluded that the mechanism of histamine release induced by X537A differs from that exerted by calcium- and energy-dependent releasing agents on the one hand and lytic agents on the other.

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Anticholeretic effect of substance P in anesthetized dogs

By

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Abstract

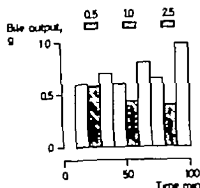
HOLM I L. THULIN and M HELLGREN *Anticholeretic effect of substance P in anesthetized dogs* Acta physiol scand. 1978 *102* 274–280.

Nine anesthetized dogs were provided with acute common duct fistulas after exclusion of the gallbladder. Synthetic Substance P was administered as caval infusions in a dosage of 0.5–20 ng kg^{-1} min 4 x 10 min. The output of hepatic bile sodium and amylase decreased during infusion by 40–52 per cent at the highest doses. After termination of infusion all 3 parameters increased by 19–60 per cent above basal level The biliary concentration of sodium was constant, while that of amylase increased during infusion. The responses were dose-related The anticholeresis induced by Substance P might be due to inhibition of the canalicular bile fraction, which presumably is mediated by active sodium transport independent of bile salt excretion

Substance P (SP) (Euler and Gaddum 1931) is an undecapeptide present mainly in central and peripheral nervous system and in the nervous structures and endocrine cells of the intestinal wall (Lembeck 1953 Pernow 1953 Amin, Crawford and Gaddum 1954, Nilsson *et al* 1975 Pearse and Polak 1975). SP has an excitatory action on motoneurons of the spinal cord (Konishi and Otsuka 1974 Henry Krnjevk and Morris 1975) and has vasodilatation (Pernow 1953 Pernow and Rosell 1975 Hallberg and Pernow 1975), contraction of smooth muscle (Euler and Gaddum 1931 Pernow 1953), and secretion of salivary glands (Lembeck *et al* 1968) and pancreas (Starke *et al* 1968). Starke *et al.* (1968) reported that crude SP extracted from intestine and brain caused contraction of the gall bladder in the dog. Hepatic bile flow was either not influenced or decreased slightly

The aim of the present work was to study the mode of action of synthetic SP on hepatic bile secretion The total bile flow was chosen as the main parameter while sodium was chosen as a substance to represent biliary electrolyte excretion. In addition amylase was allowed to represent biliary enzyme output.

Experimental evidences for 3 different mechanisms of bile secretion have been known (Wheeler 1968 Erlinger *et al* 1970, Erlinger and Dhumeaux 1974) Two fractions are of canalicular origin one being bile acid dependent and the other bile acid independent. The bile acid independent fraction is suggested to be mediated by act ... transport.



Effects of caval infusions of SP on the output of bile. ...entative experiment. Dosages: 0.5, 1.0 and 2.5 ng ... min⁻¹

...l fraction is of ductular-ductal origin and stimulated mainly by secretin, which results ...n increased bile volume and a rise in bicarbonate concentration. Small single chain ...ides, e.g. cholecystokinin, gastrin and vasoactive intestinal peptide, have been proposed ...nfluence bile flow via the electrolyte (Hardison and Norman 1968, Wheeler 1968) or ...um (Thulin and Hellgren 1976) excretion.

Material and Methods

...thetic SP was kindly supplied by Dr Karl Folkers, Institute for Biomedical Research, Austin, Texas. ...as dissolved in sterile saline, passed through a bacteria filter (Kabi, Sweden) and kept frozen until used. ...he experiments were performed in 9 medium sized mongrel dogs. Anesthesia was induced with pentothal ...um, 25 mg · kg b w⁻¹ and maintained at a steady state with repeated doses at regular intervals. No ...thotal sodium was administered 15 min prior to SP infusions. The animals were endotracheally in...ated and ventilated by an Engström respirator with $N_2O + O_2$. Mean arterial blood pressure was re...ded electromechanically via a catheter in the femoral artery. Via a small subcostal incision, the cystic ...t was ligated and a common duct drainage created close to the liver hilus. Approximately 30 min after ...gery the bile flow per 10 min was found to be constant. SP was infused in small amounts of saline into ... inferior caval vein.

...9 dogs were given a total of 23 caval infusions in dosages of 0.5–20 ng · kg⁻¹ · min⁻¹, duration 10 min, ...d bile was sampled over 10 min periods. The parameters studied were total bile output and biliary ...ntent of sodium and amylase. The bile output was measured by weighing, the sodium content by flame ...tometry and the amylase content by starch hydrolyzing capacity (Phadebas, Pharmacia, Sweden).

...Statistical analyses of the data obtained was performed by the use of Student's t-test for correlated means. ...P-value less than 0.01 was considered statistically significant.

Results

1. *Effect of SP on bile secretion*

Caval infusions of SP in doses of 1.0 ng · kg⁻¹ · min⁻¹ or higher induced a statistically significant anticholeretic effect. After termination of infusions there was a choleretic response, which however was not statistically significant at doses lower than 20 ng · kg⁻¹ · min⁻¹. Thus, at an infusion rate of 20 ng · kg⁻¹ · min⁻¹ the volume diminished from a mean control value of 1.3 g down to 0.6 g per 10 min. After termination of the infusions, the bile flow increased up to 1.7 g per 10 min (Table I). The anticholeretic effect of SP occurred within ... min after the beginning of the infusion while the post-infusion choleretic

TABLE I Effects of caval infusions of SP dosage 20 ng × kg⁻¹ × min on the output of bile, sodium and amylase p-values for change from control.

		Control	During infusion	After infusion
Bile g/10 min	$\bar{x}$	1.3	0.6	1.3
	Range	0.7–1.9	0.3–1.1	1.?–2.?
	n	7	7	7
	p<		0.001	0.01
Sodium mmol/10 min	$\bar{x}$	0.52	0.14	0.29
	Range	0.19–0.3[illegible]	0.08–0.19	0.[illegible]–0.39
	n		2	2
	p<		NS	NS
Sodium, mmol/l	$\bar{x}$	170	175	170
	Range	167–17?	171–179	165–178
	n		2	2
	p<		NS	NS
Amylase, μkat × 10⁻⁹/10 min	$\bar{x}$	4.1	2.3	7.1
	Range	[illegible].0–5.8	1.8–2.7	3.1–14.7
	n	7	7	7
	p<		0.01	NS
Amylase, μkat/l	$\bar{x}$	3.8	4.6	4.5
	Range	1.1–7.5	1.7–8.7	1.6–10.0
	n	7	7	7
	p<		0.001	NS

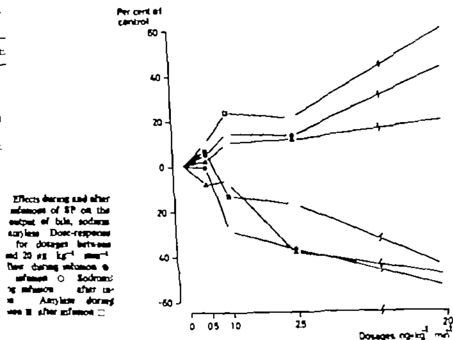
effect had a duration of about 10 min, whereafter the bile flow returned to the control level (Fig. 1). A clear dose-response relationship was demonstrated both for the anticholeretic and choleretic effect (Fig. 2)

B. Effect of SP on biliary sodium output

Caval infusions of SP in doses of 2.5 ng × kg⁻¹ × min⁻¹ or higher significantly decreased the total biliary output of sodium. At an infusion rate of 20 ng × kg⁻¹ × min⁻¹ only 2 sodium analyses were performed because of the small bile volumes obtained. During an infusion rate of 2.5 ng × kg⁻¹ × min⁻¹ the total biliary output of sodium decreased from a mean control value of 0.19 mmol down to 0.12 mmol per 10 min. After infusion the sodium output returned to slightly above the control level (Table II). A dose-response correlation was found for the effects, both during and after infusion (Fig. 2). The concentration of sodium in bile ranged by less than 8 per cent in each animal and was thus constant before, during and after infusion (Table I, Table II)

C. Effect of SP on biliary amylase output

Caval infusions of SP decreased the total biliary output of amylase, the effect being significant only at the highest dose, 20 ng × kg⁻¹ × min⁻¹. However already at a dosage of 1.0 and 2.5 ng × kg⁻¹ × min⁻¹ there was a significant increase in total amylase output after termination of the SP infusions (Table II, Fig. 2). Thus, the total biliary output of amylase decreased during SP infusions at the dosage of 20 ng × kg⁻¹ × min⁻¹ from [illegible] control value of 4



Effects during and after infusions of SP on the output of bile, sodium amylase. Dose-response for dosages between and 20 ng kg⁻¹ min⁻¹. flow during infusion ● infusion ○ Sodium: ng infusion after in- Amylase during ■ after infusion □

× 2.3 µkat 10⁻⁶ per 10 min. After the infusion period the output was 7.1 µkat 10⁻⁶ per 10 min (Table I).

The concentration of amylase in bile rose from mean control value of 3.8 to 4.6 µkat per l during infusions of SP in doses of 20 ng kg⁻¹ min. The concentration of amylase in bile during the post-infusion period varied in the different animals compared to the controls and thus no significant changes were found (Table I).

Effect of SP on blood pressure

During caval infusions of SP at a rate of 2.5 ng kg⁻¹ min and 10 min duration, there was an initial blood pressure drop from mean control value of 155 to 140 mmHg whereafter the pressure levelled off at 145 mmHg. Infusions of SP at a rate of 20 ng kg⁻¹ min⁻¹ and 10 min duration induced a pressure drop of maximally 1 min duration from a mean control value of 145 to 100 mmHg. During the continued infusions the blood pressure stabilized at a mean of 120 mmHg.

Discussion

Motor effects of SP on the extrahepatic bile ducts have been studied previously in anaesthetized dogs (Starke et al. 1968). The bile secretion was simultaneously studied after excision of the gallbladder. Crude SP-extracts of intestine and brain tissue were found to have no or only slightly diminishing effect on the bile flow. According to the authors, it could not be

TABLE II Effects of ca al infusions of SP dosage ...5 ng × kg⁻ min on the output of bile, so-
and amylase p-values for change from control

		Control	During Infusion	After infus.
Bile g/10 min	$\bar{x}$	1 1	0.7	1.3
	Range	0.7 1 7	0.4–1.2	0.9–1 1
	n	8	8	8
	p<		0 001	NS
Sodium, mmol/10 min	$\bar{x}$	0.19	0.1	0.20
	Range	0.13–0.28	0.06–0.23	0.17–0.19
	n	5	5	5
	p<		0.001	NS
Sodi m, mmol/l	$\bar{x}$	163	165	165
	Range	154–174	156–176	16. 179
	n	5	5	5
	p<		NS	NS
Amylase μ k t 10⁻⁹/10 min	$\bar{x}$	3 7	3..	4 4
	Range	1 7 5.7	1 0–6.1	2 0–7.2
	n	7	7	7
	p<		NS	0 01
Amylase μ kat/l	$\bar{x}$	3 7	4.3	4 1
	Range	1 9 5 7	..1–6.6	1 9–7 7
	n	7	7	7
	p<		NS	NS

decided whether this effect was due to a reduction of hepatic blood flow or tonus variation in the bile ducts.

In the present study SP was found to exert a biphasic action. Thus, during infusion of S there was an anticholeretic response, while after termination of infusion a choleretic respon was recorded It seems reasonable to believe that the essential effect of SP on the bile secr tion is depressive. Anticholeresis is known to occur following infusion of toxic produc (Wheeler 1968), but no physiologic substance has to our knowledge been demonstra to depress bile flow

The anticholeresis elicited by SP may be due to several factors. Some of these could considered as indirect, such as a changed liver perfusion or bile duct contraction. Oth could be considered as more direct such as enzymatic blocking of cellular synthesis transport of bile. There is no reason to believe that the anticholeresis was due to the anest tics, since SP was administered during relatively brief intervals in a steady state of gen anesthesia In fact pentothal sodium has been found to augment hepatic bile secreti (Erlinger and Dhumeaux 1974) Circulatory depressive effects of SP cannot explain the b flow reduction since even extreme hypoperfusion evidently does not affect bile flow (Ferr *et al* 1955 Fisher Lee and Fedor 1958 Brauer 1965) Furthermore SP is known to incre both hepatic arterial and portal blood flow (Hallberg and Pernow 1975). An increase tonus of the bile ducts as a cause of the anticholeresis could be ruled out by the experimen procedure used since the common duct drainage was created close to the liver hilus. Furth more, the bile canaliculi are known to be devoid of smooth muscle fibres.

The anticholeretic effects of SP might tentatively be elicited via the adrenergic or chole

stems as a result of a neurotropic effect of SP. However, it has not been possible to the hypotensive or smooth muscle stimulating effects of SP by anticholinergic or ergic blocking agents (Pernow 1953, Pernow and Rosell 1975). SP has been postulated tor function in the sensory nervous system (Konishi and Otsuka 1974) and SP mmuno-reactivity has been demonstrated in most tissues (Hökfelt *et al.* 1976). However, no such immunofluorescence has been demonstrated in the liver (Hokfelt, personal munication). An anticholeretic effect of SP exerted via nervous influences thus seems ely.

ibition of synthesis is a possible explanation for the decrease in amylase output. ever, the instantaneous effect on the amylase output provoked by SP infusions is probnot in conformity of such a mechanism. Blocking of an active transport mechanism ars to remain as a plausible explanation of the anticholeretic effect of SP. A bile fraction of canalicular origin, dependent on active sodium transport, has been demonstrated. transport is mediated by Na-K-activated adenosine triphosphatase (Erlinger *et al.*), Erlinger and Dhumeaux 1974). Inhibition of this fraction would result in a decreased l biliary sodium output and a secondarily reduced bile volume during a maintained centration of sodium in bile. This is in accordance with the findings in the present work constant concentration of sodium in bile before, during and after stimulation with SP. thermore, other small peptides with secretory effects such as cholecystokinin, vasove intestinal peptide and secretin are considered to affect choleresis by such a mechanism ardison and Norman 1968, Wheeler 1968, Thulin and Hellgren 1976). SP is also known augment the urinary output of sodium and water (Macfarlane, Mills and Ward 1974). However, from the present experiments it is not possible to exclude that both sodium and ter react secondarily to other factors such as changes in bile salt excretion. Studies on this problem are in progress.

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TABLE II Effects of [illegible] infusions of SP (dosage ~5 ng kg^{-1} min^{-1}) on the output of [illegible] and amylase p-values for change from control.

		Control	During infusion	After [illegible]
Bile g/10 min	$\bar{x}$	1.1	0.7	1.3
	Range	0.7–1.7	0.4–1.[illegible]	0.9–1.1
	n	8	8	8
	p<		0.001	NS
Sodium, mmol/10 min	$\bar{x}$	0.19	0.1	0.20
	Range	0.13–0.28	0.06–0.[illegible]	0.17–0.[illegible]
	n	5	5	5
	p<		0.001	NS
Sodium, mmol/l	$\bar{x}$	163	165	165
	Range	154–174	156–176	162–17[illegible]
	n	5	5	5
	p<		NS	NS
Amylase μkat 10^{-4}/10 min	$\bar{x}$	3.7	3.2	4.4
	Range	1.7–5.7	1.0–6.1	2.0–7.2
	n	7	7	7
	p<		NS	0.01
Amylase μkat/l	$\bar{x}$	3.7	4.3	4.1
	Range	1.9–5.7	[illegible]1–6.6	1.9–7.7
	n	7	7	7
	p<		NS	NS

decided whether this effect was due to a reduction of hepatic blood flow or tonus [illegible] in the bile ducts.

In the present study SP was found to exert a biphasic action. Thus, during infusion there was an anticholeretic response, while after termination of infusion a choleretic [illegible] was recorded. It seems reasonable to believe that the essential effect of SP on the bi[illegible] tion is depressive. Anticholeresis is known to occur following infusion of toxic [illegible] (Wheeler 1968) but no physiologic substance has to our knowledge been demo[illegible] to depress bile flow.

The anticholeresis elicited by SP may be due to several factors. Some of these [illegible] considered as indirect, such as a changed liver perfusion or bile duct contraction. [illegible] could be considered as more direct, such as enzymatic blocking of cellular syn[illegible] transport of bile. There is no reason to believe that the anticholeresis was due to the [illegible] tics, since SP was administered during relatively brief intervals in a steady state of anesthesia. In fact, pentothal sodium has been found to augment hepatic bile [illegible] (Erlinger and Dhumeaux 1974). Circulatory depressive effects of SP cannot explain [illegible] flow reduction since even extreme hypoperfusion evidently does not affect bile flow [illegible] et al 1955, Fisher, Lee and Fedor 1958, Brauer 1965). Furthermore, SP is known to [illegible] both hepatic arterial and portal blood flow (Hallberg and Pernow 1975). An inc[illegible] tonus of the bile ducts as a cause of the anticholeresis could be ruled out by the exper[illegible] procedure used, since the common duct drainage was created close to the liver hilus. [illegible] more, the bile canaliculi are known to be devoid of smooth muscle fibres.

The anticholeretic effects of SP might tentatively be elicited via the adrenergic or c[illegible]

seems as a result of a neurotropic effect of SP However it has not been possible to the hypotensive or smooth muscle stimulating effects of SP by anticholinergic or rgic blocking agents (Pernow 1953, Pernow and Rosell 1975). SP has been postulated mitter function in the sensory nervous system (Konishi and Otsuka 1974) and SP nmuno-reactivity has been demonstrated in most tissues (Hökfelt *et al.* 1976). How- no such immunofluorescence has been demonstrated in the liver (Hökfelt, personal unication). An anticholeretic effect of SP exerted via nervous influences thus seems ely

ibition of synthesis is a possible explanation for the decrease in amylase output. ever the instantaneous effect on the amylase output provoked by SP infusions is prob- not in conformity of such a mechanism. Blocking of an active transport mechanism ars to remain as plausible explanation of the anticholeretic effect of SP A bile frac of canalicular origin, dependent on active sodium transport, has been demonstrated. transport is mediated by Na K -activated adenosine triphosphatase (Erlinger *et al* Erlinger and Dhumeaux 1974). Inhibition of this fraction would result in a decreased l biliary sodium output and a secondarily reduced bile volume during a maintained entration of sodium in bile. This is in accordance with the findings in the present work constant concentration of sodium in bile before, during and after stimulation with SP thermore, other small peptides with secretory effects such as cholecystokinin, vaso- e intestinal peptide and secretin are considered to affect choleresis by such a mechanism rdison and Norman 1968, Wheeler 1968, Thulin and Hellgren 1976). SP is also known ugment the urinary output of sodium and water (Macfarlane, Mills and Ward 1974). lowever from the present experiments it is not possible to exclude that both sodium and ter react secondarily to other factors such as changes in bile salt excretion Studies on this oblem are in progress.

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Effects of the antidiuretic hormone, arginine vasotocin, theophylline, filipin and A23187 on cyclic AMP in isolated frog skin epithelium (Rana temporaria)

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Abstract

JOHNSEN, A. H. and R. NIELSEN. *Effects of the antidiuretic hormone, arginine vasotocin, theophylline, filipin and A23187 on cyclic AMP in isolated frog skin epithelium (Rana temporaria)*. Acta physiol. scand. 1978. *102*. 281–289.

[illegible] method for measuring cAMP in frog skin epithelium was developed. The epithelia were isolated after [illegible]-treatment. cAMP was extracted by boiling water and the extract was purified on dry Al_2O_3. The change with time of the cAMP level after addition of arginine vasotocin (AVT) was studied. The hormone caused a rapid increase in cAMP level with a maximum after 3–5 min, thereafter the cAMP level declined. Incubation with AVT made the epithelia refractory to a second dose of AVT, which indicates that the decline in cAMP level was caused by a feedback mechanism and not by inactivation of the hormone. cAMP appeared evenly distributed in all cell-layers of the epithelia both before and after stimulation with AVT. Theophylline caused a rapid increase in the cAMP level, which remained elevated for at least [illegible]. Addition of the ionophore A23187 or of filipin had no effect on the cAMP level. However, in the presence of theophylline, A23187 enhanced the cAMP level, whereas filipin had no effect. Therefore the involvement of cAMP in the action of A23187 has to be considered.

It is well established that the addition of antidiuretic hormone (ADH) to the inside (the blood side) increases the active sodium transport and water permeability in various epithelia, among these frog skin and toad bladder. It is generally accepted that at least some of the effects observed after addition of ADH are mediated by cAMP. One of the tissues widely used for combined measurements of permeability changes and cAMP is the toad bladder and a number of reports have shown that ADH enhances the level of adenosine 3',5'-cyclic monophosphate (cAMP) in toad bladder (for references see Andreoli and Shafer 1976).

The time course of cAMP level following addition of ADH has been investigated in toad bladder. Mendoza *et al.* (1972) found the same cAMP levels 10 and 60 min after addition of ADH, and Flores *et al.* (1974) found that the cAMP level reached a steady state level 10 [illegible] after the addition of ADH and stayed there for at least 30 min, whereas Sapirstein and [illegible]) found that [illegible] level reached a maximum after 3–6 min and then declined.

Concerning the effect of ADH on the isolated frog skin we know of only 2 reports. Shorderet Grosso and de Sousa (1975) found that incubation of isolated epithelia ... ADH (oxytocin) plus 1 mM theophylline enhanced the cAMP level. 2) In their review ADH Jard and Bockaert (1975) referred to unpublished data according to which addition ADH (oxytocin) in isolated epithelia resulted in a transient increase in the cAMP level. (The last two reports did not present methods or figures.)

The purpose of this study has been to develop a method for measuring cAMP in the skin epithelium and to investigate the changes in cAMP level induced by ADH and theophylline. Furthermore it was investigated whether the activation of active sodium transport induced by filipin (a polyene antibiotic) and A23187 involved cAMP.

Materials and methods

Methods

The experiments were performed o male and female frogs (*Rana temporaria*). The frogs were kept partly immersed in tap water at about 4 C.

It is well known, that there is a p onounced season l and biological ... ration in the different ... parameters. Concerning cAMP Hall *et al.* (1976) found a considerable d fference between the cA... levels ... *hole skins* from winterfrogs and summerfrogs. The individual experimental series of the present communication were performed in d fferent seasons, so it is to be expected that the cAMP levels ... under elsewise equal condition will vary from one series to the other. Therefore most of the expts. ... perf rmed o symmetrical halves, ne half serving as control of the other.

The skins were mou ted i perspex chambers (rea 7 cm²) (Ussing and Zerahn 1951) and bathed in ... fied Ringer's solut on (N ... 115 K ... 5 Ca^{++} 1 Mg^{++} 1 Cl^- 116, CO_3^{2-} 2.5 SO_4^{2-} 1 PO_4^{3-} 1 phosphate mM pH = 7.8). This solution was used for all incubations and referred t as "medium". The epithelia were isolated by collagenase treatment of the inside a described fo *Rana esculenta* by Rajerison *et al.* (19..) with the follow g mod fications 1) The sk ... w s ncubated without hydrostatic pressure and 2) at room temperat re instead of 35 C, as the kin of *R. temporaria* did not survive at the higher temperature. After 1 1.5 h of incubation with collagenase the skin was washed twice, nd when desired the skin was cut ... of the chamber and placed on a glass plat and the intact epithelium was scraped off by means of a microscope slide. After the incubation (see below), cAMP was extracted by dropping the epithelium into 500 ... of destilled wat r co tain ng H-cAMP as recovery marker in a small test t be (pyrex glass, 75 ... mm) placed in a boili g water bath. The t be was capped and 7 min later it was transferred to a icebath. The content was homogenized 2 1 m n with rotat g teflon pestle that f tted loosely the test tube. The homogenate was centrifuged for 10 min at 10 000 *g* m the same tube. 400 μl of the supernatant ... placed on a minicolumn of 1.8 g dry Al_2O_3 n Pasteur pipett and eluted w th ml destilled ... ater. The eluate was lyophil zed, dissolved buffer nd cAMP wa measured by a binding protein assay (Gerber *et al.* 1977) in duplicates at two dilutio s.

The protein-precipitat which remained after the boil ng and centrifugation was dissolved by boiling 10 min in 1 ml 1 M N OH and the protei was measured by biuret method usi g bovine serum albumin (also d ssolved in 1 M N OH and boiled 10 min) as standard.

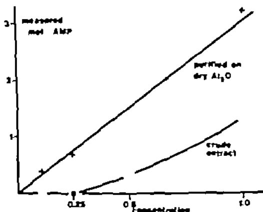
The effects of the various drugs on the epithelia were tudied in two ways.

1) After incubation with collagenase the epithel um was isolated as described abo and transferred to a test tube (polyethylen, 100 15 mm) co ta ni g 5 ml medium. The test tube was placed in ... test tube rotator and equilibrated f r 1 2 h after which th periment was carried o t under the desired conditions and cAMP was extracted as described bove.

2) When exposure to drugs on ly one side was desired (*i.e.* incubation with fil pin and A23187) the procedure was a little d fferent. After collagenase treatment the whole skin remained in the chamber, and incubation took place here. Thereafter the skin was cut t, the epithelium scraped off and cAMP was extracted.

When theophylline was included in the med um th sod m chloride content was reduced proportionally t keep the osmolarity constant s measured by freezing-point depression.

The short circuiting expts. were performed as described by Ussing and Zerahn (1951).



An extract of frog skin epithelium, prepared as described in the text, was divided in two. One half remained untreated and the other was purified on dry Al_2O_3, and cAMP was measured at 4 dilutions in both.

[Results of extraction and purification procedure]

[Adsorption] of cAMP on Al_2O_3 was originally described for separation of cAMP and ATP (the latter being [completely] adsorbed [while] cAMP is not adsorbed) in an adenylate cyclase assay (Ramachandra 1971). [We] found that the method was also sufficient for removing substances that interfered [with] the cAMP [assay], when it was performed on the crude water extract, as show[n] in F[i]g. 1.

[The] extraction of cAMP [with] boiling [wa]ter described above [was] compared to freeze-clamping in liquid [N_2] followed by extraction of cAMP in ice-cold trichloroacetic acid (TCA) and purification on dry Al_2O [(the] latter demanded ether-extraction of the TCA-extract, lyophilisation and redissolving in water). After [incubation] with arginine vasotocin (AVT) alone we found much higher cAMP levels in the [w]ater extract [than in] the TCA extract (Table I). This difference could have 2 explanations, either that the adenylate [cyclase] was activated by the heating or that the phosphodiesterase [w]as not destructed fast enough by TCA [extraction]. That preincubation [w]ith 30 mM theophylline eliminated the difference (T[a]ble I) excluded the [first] explanation and the boiling method was chosen.

[It] has been reported that the activity of phosphodiesterase (from beef heart) [a]t 0°C [w]as more than one [fifth] of the activity at 20°C (O'Dea, Haddox and Goldberg 1970). Therefore the reason [wh]y the com[bined TCA] and freeze-clamping technique failed, might be that the penetration of TCA during thaw[in]g in ice-[cold] TCA was too slow due to the thickness of the epithelia which rolled up [w]hen they were lifted out of [the] incubation media.

Materials

[Arginine]-vasotocin (AVT), the neurohypophyseal hormone from amphibia (a gift from Sandoz) [w]as used [in a] final concentration of 40 ng/ml.

Theophylline was obtained from Sigma.

[Pilo]carpin (a gift from the Upjohn Company) was used in [a] final concentration of 50 μM.

[A]23187 (a gift from Eli Lilly & Co) was used in [a] final concentration of 4 μM.

Al_2O (BDH and Merck) was in the neutral form.

[Table] I. Extraction of cAMP from the epithelia [w]ith boiling [w]ater or TCA. Paired halves were incubated under the conditions given above. One half was freeze-clamped in liquid N_2 and cAMP extracted by ice-cold TCA. cAMP in the other half [w]as extracted by boiling [w]ater. Values are pmol cAMP/[m]g protein ± S.E.

[Treatment]	TCA	Boil. H_2O	Δ	
[None]				
5 min AVT	1.79±0.04	1.75±0.10	−0.04±0.09	4
30 min theo	5.16±0.71	11.37±2.17	6.21±1.41	6
5 min (theo + AVT)	19.76±2.65	19.31±2.71	−0.46±1.50	6

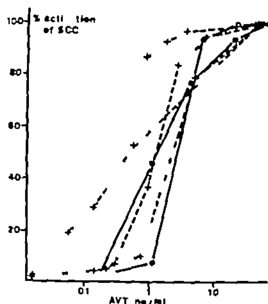


Fig. 2. Normalized (cumulative) response of SCC increasing AVT-concentration. Each expt was conducted on paired hal es, one remaining unstimulated as control to compensate for time-dependent [illegible] of basal SCC. At start of expt. a b. Per cent activation of SCC as calculated as follows: % activation of SCC = (a − b)/c 100, where a SCC of experimental half b = SCC of control half c maximal AVT-induced increase of SCC. +—+ 4 separate expts. with untreated skins, O—O, 2 separate expts after preincubation with collagenase.

Collagenase (C. histolyticum) was obtained from Boehringer. Grade I was used in a final concentration of 135 mU/ml and grade II in a concentrati n of 45 mU/ml. Grade I was only used in half of the experiments with theophylline and filipin; the reason for replacing grade II with grade I in this series was that incubation with grade II w s found to diminish the activation of SCC by filipin considerably, while incubation with grade I d d not (data not shown here).

Results

Effect of collagenase on AVT stimulation

It has been shown that the epithelia of *R. esculenta* preserve transporting capacity and responsiveness to stimulation by neurohypophysial hormones after incubation with collagenase (Rajerison *et al.* 1972).

To test our preparation we compared the effect of AVT on the short-circuit current (SCC) of paired skin halves, one of which had been preincubated 1 h with collagenase. Without collagenase the maximal increase of SCC following stimulation with AVT reached [illegible] above the resting level and with collagenase preincubation 49 [illegible] (mean of 4 expts.).

We also tested the response to increasing doses of AVT (*i.e.* a cumulative dose-response). Curves from skins which had been preincubated 1 h with collagenase and from untreated skins were almost identical when per cent of maximal response was used as ordinate (Fig. 2).

These findings indicate that preincubation with collagenase (grade II) destroyed some of the AVT-receptors in the epithelium but had no effect on the remaining functional receptors.

Time-course of the response to AVT

The concentration of AVT (40 ng/ml) used in these expts. caused maximal stimulation of the SCC (Fig. 2). Paired halves were used for each measurement, one was kept unstimulated as control and the other incubated with AVT for the desired length of time. As it appeared that the levels of the controls varied fairly little in this series (1.92 ± 0.11 pmol cAMP per [illegible] protein (mean ± S.E., n = 15)) and were substantially smaller [illegible] stimulated levels

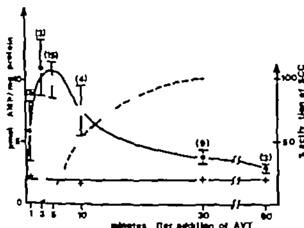


Fig. 3. Effect of AVT on the cAMP level in isolated frog skin epithelium. cAMP was extracted after the indicated time of incubation: ●—● cAMP level in the presence of AVT mean ± S.E., figures in parentheses number of expts. +—+ cAMP in control epithelia. ---- typical increase in SCC after addition of AVT increase after 30 min of incubation = 100 %.

...ol halves were omitted in part of the measurements. After addition of AVT there was ...ed increase in the cAMP level, which reached a maximum in 3–5 min and thereafter ...ined, reaching nearly the control level after 60 min (Fig. 3) The broken line in Fig. 3 ...s a typical response of SCC after addition of AVT

The fall in cAMP after 5 min in spite of continuous exposure to AVT could be due to ...dation of the hormone. As seen from Table II epithelia which had been incubated ...h AVT for 60 min showed only a small increase in cAMP level 5 min after the addition ...a second dose, and the cAMP level reached was far below that observed in epithelia only ...osed to AVT for 5 min. This indicates that the observed decrease in cAMP level (Fig. 3) ...uld not be explained by degradation of AVT

...relations between cAMP and epithelial protein content

...e frog skin has a multilayered epithelium consisting of 3–7 cell-layers, and as we always ...ed the same area of the epithelia, it seems reasonable to assume that the amounts of pro... measured were proportional to the number of cell-layers. To examine how cAMP was

...LE II. Effect of AVT on the cAMP level after 60 min preincubation with AVT. In the first series paired halves were incubated 65 min with AVT and a fresh dose of AVT was added to one half for the last 5 min. In the second series of paired halves one half was incubated 60 min with AVT and the other half remained untreated, after the 60 min period a fresh dose AVT was added to both halves for 5 min

...cubation ...h AVT, min	pmol cAMP/mg protein ± S.E.	Δ	
...0	0.84 ± 0.24		
... 5 / ...–5	1.44 ± 0.40	0.61 ± 0.17	3
	2.05 ± 0.35		
...5	5.61 ± 1.10	3.73 ± 1.34	4

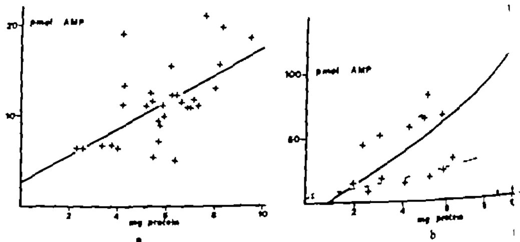


Fig. 4 a. Correlation between the amounts of cAMP and protein in unstimulated epithelia pmol cAMP = 1.51 mg protein + 2.61 r = 0.59 p < 0.001 Intercept of y-axis is not significantly different from 0.

Fig. 4 b Correl tion between the amounts of cAMP and protein in epithelia incubated 5 min with ... pmol cAMP = 12.27 mg protein − 10 79 r = 0.68, p < 0.01 Intercept of y-axis is not significantly different from 0, ... − 15

distributed in the epithelia the total amounts of cAMP were plotted against the amounts of protein. The points obtained from unstimulated epithelia showed a significant correlation (Fig. 4 a) Those from epithelia incubated 5 min with AVT also showed a significant correlation (Fig. 4 b) But looking at the latter figure it seemed as if the results fell into 2 groups, the regression lines of which are drawn as dashed lines The deviations from zero of the slopes of both lines are significant (upper line p < 0 01 lower line p < 0.05) and so is the difference between the slopes (p < 0 02) The explanation remains obscure for the time being.

Time-course of response to theophylline

Theophylline is an inhibitor of the enzyme phosphodiesterase, which hydrolyses cAMP and it is known to mimic the effects of ADH (Orloff Handler and Preston 1962) and cause an accumulation of cAMP in toad bladder (Handler *et al* 1965)

In order to study how the cAMP level changed with time, paired halves were incubated for different periods with 10 mM theophylline Three series were made. In the first series one half was incubated 1 5 min and the other half 5 min, in the other series incubation times were 5 and 15 min, and 15 and 45 min

cAMP reached a new steady state level of 7 66 ± 0.33 pmol cAMP/mg protein (mean ± S E., n = 8) within 5 min and this level was maintained for at least 45 min A few frogs responded considerably stronger than the major part and was omitted from the calculation above.

Effects of filipin and A23187

Both filipin and A23187 cause an activation of the SCC. Therefore it was investigated whether these drugs also had an effect on the cAMP level.

The addition of filipin or of A23187 alone to the inside had no effect on cAMP level (Table III) However in the presence of 10 mM theophylline A23187 increased the cAMP level whereas filipin had no effect (Table IV)

III Effect of filipin and A23187 on cAMP level. Paired halves were used. One half was kept as control while the other half received the ionophore for 10 min. Values are pmol cAMP/mg protein ± S.E.

	Experiments	Control	Δ	
A23187 on inside	3.36 ± 0.27	2.90 ± 0.39	0.46 ± 0.32	5
filipin on inside	3.53 ± 0.18	3.17 ± 0.32	0.36 ± 0.30	5

Discussion

...der *et al.* (1965) found that ADH increased the cAMP level in toad bladder and thereby ...orted the hypothesis that the effects of ADH are mediated by cAMP. In the present ...munication it has been shown that the addition of the amphibian antidiuretic hormone, ... also caused an increase of the cAMP level in the frog skin epithelium (Fig. 3).

...he swift increase in cAMP level following addition of AVT is contrasted by the consider... slower rise in SCC (Fig. 3). This finding is in accordance with the view that the physio...cal effects of ADH are not functions of cAMP concentration as such, but rather of the ...ree of phosphorylation of some membrane proteins, which again should be regulated by ...MP (Walton *et al.* 1975). Furthermore Fig. 3 shows that the increase in cAMP level was ...wed by a second phase with a fairly rapid decline. This raises the question how the ...ease, observed during continuous exposure to AVT, was brought about. From Table ... is seen that the response of the epithelium to a second dose of AVT was small after 60 ... incubation with AVT. Likewise Huja and Hong (1976), who measured SCC, found that ...d skin remained refractory to ADH for at least 2 h after the first dose had been washed ... Thus AVT appears to elicit a negative feedback with a considerable delay time and after... ect. Two explanations of this phenomenon seems possible. Either that the breakdown of ...MP by phosphodiesterase is enhanced or that the synthesis by adenylate cyclase is re...duced. The second explanation is supported by the finding that the adenylate cyclase ... pig kidney became desensitizised during incubation with ADH (Roy, Guillon and ...rd 1976).

Unlike the cAMP response to AVT, the cAMP level following addition of 10 mM theo...phylline stayed elevated after a rapid increase. If the decline of the cAMP level in the second ...ase of stimulation with AVT is due to a lowered adenylate cyclase activity, then the lack ...f decrease in cAMP level after stimulation with theophylline indicates that the feedback ...s no effect on the unstimulated adenylate cyclase, but only has an effect on the hormone

Table IV Effect of filipin and A23187 on cAMP level with 10 mM theophylline present. Paired halves were preincubated 15 min with 10 mM theophylline. One half received the ionophore for additionally 20 min, while the control half remained with no further additions. Values are pmol cAMP/mg protein ± S.E.

	Experiment	Control	Δ	
5 µM A23187 on inside	23.99 ± 3.50	14.93 ± 4.87	9.06 ± 1.48	4
5 µM A23187 on outside	10.00 ± 2.30	6.16 ± 0.87	3.84 ± 1.94	4
20 µM filipin on inside	13.54 ± 2.13	13.41 ± 2.20	0.13 ± 1.09	9

stimulated enzyme activity The reason might be that a cAMP induced feedback mechanism uncouples a step between hormone binding and cAMP production. Or the feedback could be triggered from an element in the chain of events leading to stimulation of the adenylate cyclase

Using compartmental analysis Morel and Leblanc (1973) claimed that sodium enters from the outside only into the outermost living cell-layer (first RCL) during short-circuit conditions. It has also been shown that ADH caused swelling merely of the first RCL (Voûte and Ussing 1970) This indicates that it is only the first RCL which responds to ADH This raises the question whether the hormonal stimulation of cAMP level only occurs in the first RCL. If this were so the total amount of cAMP in stimulated epithelia should be rather independent of the number of cell-layers, while on the other hand the total amount of cAMP should be proportional to the number of cell-layers if they were stimulated by AVT From Fig. 4 a it seems reasonable to conclude that cAMP in non-stimulated epithelia is distributed evenly in all cell layers of the epithelium. From Fig 4 b it seems likely that also the increase in the cAMP level elicited by AVT takes place in more than the first RCL.

Both the polyene antibiotic filipin (Nielsen 1977 a) and A23187 (Nielsen 1976) caused an increase in SCC when added to the inside of frog skins. This effect of filipin was abolished by preceding treatment with AVT (Nielsen 1977 b) This was not due to a maximal stimulation of SCC by AVT since it was possible to activate the SCC further by diphenylhydantoin. Thus the activations of SCC by AVT and filipin may be mediated by the same mechanism. With the aim of elucidating a possible causal relationship between cAMP and SCC the effects of filipin and A23187 on the epithelial level of cAMP were investigated.

Neither filipin nor A23187 caused an increase in cAMP level by themselves (Table III). This indicates that the activation of SCC by filipin and A23187 does not involve cAMP and more generally that an elevation of the cAMP level is not a necessary prerequisite for activation of SCC. Then the possible common step in the action of AVT and filipin or A23187 must be at a point later than cAMP production.

With theophylline in the medium filipin did not cause an increase in cAMP level but A23187 did (Table IV) This may indicate that there is a difference—quantitative or qualitative—in their action, which is supported by the observation that the effect of filipin on SCC was abolished by preceding incubation with A23187 while preceding incubation with filipin did not abolish the stimulation of SCC by A23187 (Nielsen 1977 b)

That an elevation of the cAMP level was only measured with theophylline present, indicates that A23187 only caused a very small increase in the cAMP production. Localized increases in cAMP level may bring about physiological effects, but be undetectable unless the decomposition is reduced by theophylline. Still, the increase in cAMP level following A23187 in the presence of theophylline may be secondary to a primary effect of A23187 as ionophore, since divalent cations are known to affect the activities of adenylate cyclase (Bockaert Roy and Jard 1972) Christophe *et al* (1976) found that A23187 increased cGMP level in guinea pig pancreatic acinar cells, and ascribed this to an effect of the increased calcium level Thus it may be that A23187 enhanced cellular concentration of the divalent cations (magnesium or calcium) and thereby caused an increase level.

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stimulated enzyme activity The reason might be that a cAMP induced feedback mechan uncouples a step between hormone binding and cAMP production. Or the feedback coul be triggered from an element in the chain of events leading to stimulation of the adenyla cyclase

Using compartmental analysis Morel and Leblanc (1973) claimed that sodium enters from the outside only into the outermost living cell-layer (first RCL) during normal sho circuit conditions. It has also been shown that ADH caused swelling merely of the fi RCL (Voûte and Ussing 1970) This indicates that it is only the first RCL which respo to ADH This raises the question whether the hormonal stimulation of cAMP level occurs in the first RCL. If this were so the total amount of cAMP in stimulated epithel should be rather independent of the number of cell layers, while on the other hand t total amount of cAMP should be proportional to the number of cell-layers if they were stimulated by AVT From Fig. 4 a it seems reasonable to conclude that cAMP in stimulated epithelia is distributed evenly in all cell-layers of the epithelium. From Fig 4 seems likely that also the increase in the cAMP level elicited by AVT takes place in m than the first RCL.

Both the polyene antibiotic filipin (Nielsen 1977 a) and A23187 (Nielsen 1976) caused increase in SCC when added to the inside of frog skins. This effect of filipin was abolished by preceding treatment with AVT (Nielsen 1977 b) This was not due to a maximal stim tion of SCC by AVT since it was possible to activate the SCC further by diphenylhydan Thus the activations of SCC by AVT and filipin may be mediated by the same mechan With the aim of elucidating a possible causal relationship between cAMP and SCC effects of filipin and A23187 on the epithelial level of cAMP were investigated.

Neither filipin nor A23187 caused an increase in cAMP level by themselves (Table This indicates that the activation of SCC by filipin and A23187 does not involve cA and more generally that an elevation of the cAMP level is not a necessary prerequisite activation of SCC. Then the possible common step in the action of AVT and filipi A23187 must be at a point later than cAMP production

With theophylline in the medium filipin did not cause an increase in cAMP level A23187 did (Table IV) This may indicate that there is a difference—quantitative or qua tive—in their action, which is supported by the observation that the effect of filipin on was abolished by preceding incubation with A23187 while preceding incubation with fi did not abolish the stimulation of SCC by A23187 (Nielsen 1977 b)

That an elevation of the cAMP level was only measured with theophylline prese dicates that A23187 only caused a very small increase in the cAMP production. Loca increases in cAMP level may bring about physiological effects, but be undetectable u the decomposition is reduced by theophylline. Still the increase in cAMP level follo A23187 in the presence of theophylline may be secondary to a primary effect of A2 as ionophore, since divalent cations are known to affect the activities of adenylate cy (Bockaert Roy and Jard 1972) Christophe *et al* (1976) found that A23187 increased c level in guinea pig pancreatic acinar cells, and ascribed this to an effect of the incr calcium level. Thus it may be that A23187 enhanced cellular concentration of the di cations (magnesium or calcium) and thereby caused an increase evel.

...he ribosome content of erythroid cells provides a good index of the degree of cellular ...ation. In a transmission electron microscopy study Yamamoto and Iuchi (1976) ... an increased electron-opacity of the cytoplasmic matrix due to accumulation of ...globin concomitant with the disappearance of the cytoplasmic organelles.

...present only few data concerning the kinetics of the maturation of erythroblasts into ...erythrocytes and the synthesis of hemoglobin during the maturation in the circulating ...of fish are available. In the present investigation an attempt has been made to establish ...data in salmon (*Salmo salar* L.) by developing a method based on the incorporation ...e into hemoglobin.

Material and methods

...material and preacclimatization. 3–6 months old salmon (*Salmo salar* L.) (mean weight ± S.D. ... 2.15; range, 2–9 g) supplied by the Fishery Board from the breeding plant and salmon hatchery in ...Uppland, Sweden ...ere used. The fish were preacclimatized for two weeks to running, aerated ...tap ...ater. This originally carbonated ...ater derives from an esker stretching in some parts through ...with limestone deposits. It ... poor in oxygen and has ... fairly high but constant ion composition, ... 2.0–2 ... mmol/l; Mg^{2+} 0.5–0.6 mmol/l, and HCO... ...bout 5 mmol/l according to Höglund *et al.* ... The fish ...ere fed daily ...ith pellets (Salmon starting pellets, Astra Ewos AB, Södertälje, S...eden). ...experiment ...as performed during October–December. During the experimental period the water ...erature dropped from about +12 to +10°C.

...group of ... year old salmon supplied by the Salmon Research Laboratory at Älvkarleby ...ere used ... whole-body autoradiographic study. These fishes were kept under identical environmental conditions ... 3–6 months old ones as described ...bove.

...ministration of ⁵⁹F... and preliminary whole-body autoradiography. The fishes were anesthetized with ...222 (Sandoz, Switzerland) and each specimen was given 1 µCi of ⁵⁹F... (1–0.1 µg F... as ferric citrate ... 1 ... sodium citrate and made isotonic ...ith sodium chloride, Amersham England 1971 code ...29). This amount was injected into the peritoneal cavity as ... single dose.

...travenous injection may easily cause bleeding ...hen handling small specimens. In order to ensure that ...ble dose of ⁵⁹F... administered i.p. was incorporated in the iron stores of the salmon, *i.e.* into the liver ...en, and kidneys, and to get an estimation of the time factor for this incorporation, ... preliminary test ... carried out ...ith 2 year old salmon. Equivalent doses of ⁵⁹F... were injected i.p. in one group and i... ...the other group. Three days later the fish were killed and ...hole body autoradiography ...as performed ...described by Ullberg (1954, 1958).

...lood sampling and preparation of blood smears. Day 0 the fishes were given ⁵⁹F... i.p., and blood samples ...taken 1, 6, 10, 15, 20, 41 and 52 days later. The fish were killed by ... blow on the skull and blood ...drawn from the dorsal aorta in heparinized capillaries after transection of the caudal fin. T... blood ...ears from each fish were fixed on glass slides ...ith 1% glutaric aldehyde in isotonic solution (Fowler ... 1969). The isotonic solution contained per litre NaCl 6.00 g, KCl 0.20 g, $MgCl_2$–$6H_2O$ 0.10 g, $NaHCO_3$ ... g, $CaCl_2$ 0.20 g, NaH_2PO_4–H_2O 0.05 g, glucose 1.00 g, pH 7.3–7.4.

...utoradiography of blood smears. All blood samples fixed on glass slides were processed simultaneously ...in glass slides were dipped into ... mixture consisting of equal portions of nuclear emulsion (Ilford K5) ...d 1% glycerol in ...ater. The preparations were exposed ... 0–4°C for 15 days and then developed ...ith ...odak D19B. The RBC ...ere finally stained in the solution of Giemsa (Azure–Eosine–Methylene blue) ...0.05 M phosphate buffer at pH 7.2.

...Microscopic examination and classification of RBC. Light microscope examination of the RBC was per...formed at the magnification of 720 times. The background grain counts were obtained by counting areas ...of the same size as the RBC. Ten such areas equally divided over the investigated part of the preparation ...ere counted in each smear. A part of the background grain count derives from the ⁵⁹Fe bound to plasma ...teins. As the background grain count was small compared to the grain count of the RBC the plasma ...⁵⁹Fe has been neglected.

The RBC were classified according to the morphological criteria shown in T...ble I. This classification ...as based on the developmental series proposed by Catton (1951), Topf (1955), and Weinberg *et al.* (1972). ...Thereafter the silver grains in the nuclear emulsion laying above the cell were counted and the long and the

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Maturation of circulating red blood cells in young baltic salmon (Salmo salar L.)

By

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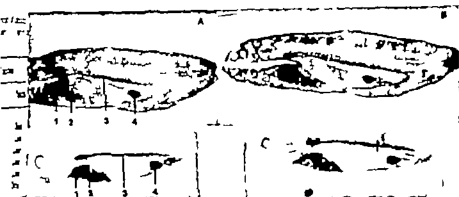
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Abstract

HÄRDIG J *Maturation of circulating red blood cells in young baltic salmon (Salmo sal*
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The maturation of circulating red blood cells (RBC) in salmon (*Salmo salar* L.) has been studied A de mental RBC series of 6 classes based on morphological criteria was proposed After a single dose (⁵⁹Fe) given to 5–6 months old salmon the incorporation of radioactivity in maturing RBC was la by utoradiography on blood smears. The relative distribution of labelled RBC between the 4 di specimens taken 15, 20, 41 and 5 days after the injection of iron showed that the RBC matured alo proposed series. The projected area of the RBC increased by 100% during the maturation. The growth was caused by an elongation of the RBC. Thus the ratio between short and long axes of the decreased with maturation nd can be used as a measure of the degree of maturation. The uptake of active iron measured as grain count per RBC or per projected area of the RBC increased during the ma tion in classes II IV Radioactive iron was not incorporated by mature RBC. The maturation time class I to class VI was shorter than 41 days. RBC iron bound to non-haemoglobin proteins as a p restriction in estimations of the haemoglobin concentrations of developing RBC is discussed.

Fish blood contains red blood cells (RBC) of different size and shape (Catton 1951, Se and Beams 1969 Yamamoto and Iuchi 1976) The RBC are released into the vascular s as comparatively small circular cells. As development proceeds the size increases an RBC become more elongated or elliptical in form as described by Topf (1955). Acco to Topf the release occurs at the erythroblast stage (in the carp, *Cyprinus carpio* L.) the synthesis of haemoglobin does not start until a stage designated proerythroc reached. The whole cell as well as the nucleus of the proerythrocyte are elliptical in co to the more circular erythroblast with a rounded nucleus (Topf 1955). Furthermore on histological findings Catton (1951) has distinguished young RBC from older on teleost fishes calling the round RBC reticulocyte Other changes in the maturating RBC have been shown by Sekhon and Beams (1969) who found that polyribosomes mitochondria were relatively numerous in the immature RBC, but decreased in nu concomitantly with RBC maturation. These findings were verified by Yamamoto Iuchi (1976) in their studies on erythrocytes in developing rainbow d they sugg



Photographs of original sections (above) and autoradiograms (below) of two-summer old *Salmo* ... injected three days earlier with ^{59}Fe: A, intraperitoneally; B, intravenously. 1 heart, 2 liver, 3 kidney ...

... morphological changes of the RBC from classes I–VI are characterized mainly by ... ing long axes and to some extent by decreasing short axes. This results in an increased ... ected area of the RBC from classes I–VI and a decreasing short/long axes ratio (Table II). ... The CV calculated for the mean values of ratio were lower in all classes as compared to ... corresponding CV for the mean values of projected area (Table II). Therefore it is ... ested that the ratio is preferable to the projected area as an index when morphometry ... ed as classification of developing RBC.

... he frequency of labelled RBC in the proposed age classes of RBC is shown in Fig. 3 ... lled RBC per class are expressed as a percentage of all RBC (labelled nonlabelled) ... n the four sampling occasions in Fig. 4. As all calculations are based on 4 and in one ... e (day 41) 3 specimens no statistical calculations except mean values were performed. ... can be seen in Fig. 3 that the frequency of labelled RBC has a maximum in class III day ... n class IV day 20, class V day 41 and class VI day 52. These successive changes with



Fig. 2. Typical autoradiographic preparation of blood smear from blood sampled 52 days after the injection of single dose of ^{59}Fe.

TABLE I Classification and morphological characteristics of circulating red blood cells (RBC) in [illegible] salmon. The cells are f[illegible]ed with glutaric aldehyde in isotonic solution and stained [illegible] solution.

Age class of RBC	Shape of cell projection	Appearance of nucleus		Stained cytoplasm colour
		Outline	Structure	
I	Circular	Circular	Dense	Light blue
II	Circular	Circular	Loose	Pale red
III	Slightly elliptical	Slightly elliptical	Loose	Red
IV	Slightly elliptical	Elliptical	Loose	Red
V	Elliptical	Elliptical	Loose	Red
VI	Elliptical	Elliptical	Dense	Red

short axes of the cell were measured by means of an ocular scale in the microscope. Smears from [illegible] were examined from day 15 [illegible]0, and 52 and 3 fishes from day 41 [illegible]0 to 40 cells in each class [illegible] The ratio between the short and the long axes of individual RBC, i.e. short/long axis index, [illegible] as well as the projected area of each cell assuming a elliptical projection. On 30 RBC from each [illegible] short and the long axes of the nucleus as well as the whole RBC were measured and the projected [illegible] the nucleus, the whole cell, and the cytoplasm were calculated. All microscopic investigations were per[illegible] formed by one person.

Presentation of quantitative data and statistical treatment The 6 age classes of RBC are labelled [illegible] Roman numerals (I VI). The quotient between the short axis and the long axis of the RBC is [illegible] the short/long [illegible]xes ratio.

Mean values of cellular morphometry given are followed by SE and coefficient of variation (CV). [illegible] regression lines were calculated using least squares [illegible]nd the coefficients obtained [illegible]ere tested using Stude[illegible] T test.

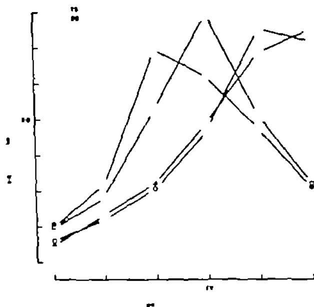
Results

The result of the whole-body autoradiography is exemplified in Fig. 1 In both cases th[illegible] radionuclide appears in liver spleen, kidney and in vascular organs. On the basis of the[illegible] results I p injection was used in subsequent expts.

Fig 2 shows a typical part of an autoradiographic preparation of RBC used for th[illegible] examination of radioactively labelled RBC. All over the emulsion there are tracts of back[illegible] ground radiation Generally the grain counts of labelled cells were 3–4 times the backgro[illegible] RBC were scored as labelled when their grain count was estimated to be twice the numb[illegible] of background grains. In a few fish the RBC showed extremely low incorporation, proba[illegible] due to unsuccessful injection of radionuclide Such specimens have been excluded.

The presentations in Table II and in Fig 3–7 do not include counts from early parts [illegible] the tests, because there are no labelled cells in the autoradiographs from days 3 and 6 af[illegible] the injections and after 10 days there are still few labelled RBC.

Results from morphologic measurements of the RBC in the different classes are sho[illegible] in Table II The mean values of short and long axes have been used to present the "mea[illegible] cell in each class. The results from the measurements of the RBC nuclei are also present[illegible] in Table II The ratio between the short and the long axes as well as the projected area [illegible] the RBC and the nucleus assuming them to be elliptical are given The relative project[illegible] areas of whole RBC and of cytoplasm has been calculated from each class with refere[illegible] to the mature RBC of class VI



) Relative distribution of lab-d RBC in age classes I to VI Table I) 13, 20, 41, and 52 after single dose of ^{59}Fe by peritoneal injection.

Since unlabelled as well as labelled RBC were found in all classes except in class III, day when the specimens were examined, the percentages of labelled RBC of all RBC in each were counted as shown in Fig. 5. The highest percentages of labelled RBC were found classes III–V. The low labelling per cent for class VI in all curves in Fig. 5 is due to the fact that this class consists of mature RBC and only a small amount of labelled RBC (compared with the mature pool) which have matured during the experimental period. Class V

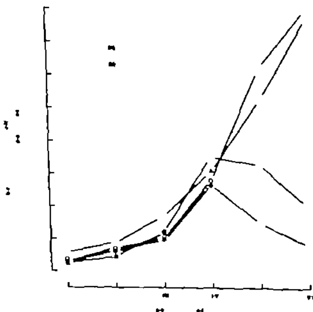
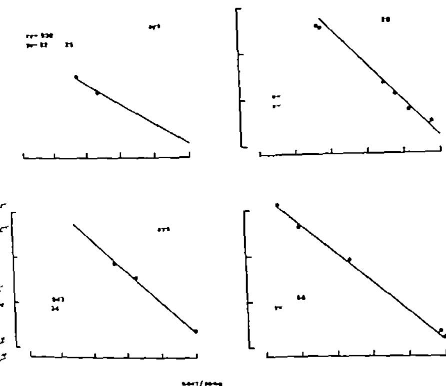


Fig. 4. Labelled cells in each age group I to VI (see Table I) as percentage of all RBC examined. The graphs represent the situation 13, 20, 41 and 52 days after single dose of ^{59}Fe by intraperitoneal injection.

Table II Morphometry of young salmon RBC. The measures of cellular and nuclear [illegible] and the ratio of short/long axes as well as the projected area of the cell, nucleus and [illegible] for 6 proposed age or maturation classes of RBC. Cell mean values, SE and CV [illegible] and methods pp 291) are based on all RBC examined, nucleus and cytoplasm [illegible] 30 measured cells in each class.

Age class of RBC		I	II	III	IV	V	VI
Cell							
Short axis (μm)	M	6.9	7.2	6.8	6.7	6.6	[illegible]
	SE	.28	.30	16	13	.13	[illegible]
	CV	4 1	4.2	2.4	2.0	1.9	[illegible]
Long axis (μm)	M	7 7	9 1	10.0	10 9	12 1	[illegible]
	SE	.29	.23	15	12	11	[illegible]
	CV	3 7	2.6	1.5	1 1	.9	13
Ratio	M	90	.80	.69	.62	.55	[illegible]
	SE	.01	02	.01	.01	.01	[illegible]
	CV	.89	75	.58	.65	.53	[illegible]
Area (μm²)	M	41	51	54	57	63	[illegible]
	SE	77	69	.54	40	.39	[illegible]
	CV	1 9	1.3	1.0	7	.6	[illegible]
Relative RBC area		63	78	81	87	96	[illegible]
Nucleus							
Short axis (μm)	M	4 6	4 7	4 6	4.4	4 0	[illegible]
	SE	.09	11	08	10	[illegible]	[illegible]
	CV	1 9	2.4	1 7	2.2	2.1	[illegible]
Long axis (μm)	M	4.8	5 4	5.9	5.9	6.3	[illegible]
	SE	10	11	10	.09	.07	[illegible]
	CV	2.1	2.1	1 7	1.5	1.1	[illegible]
Ratio	M	.96	87	79	75	.63	[illegible]
	SE	02	02	.01	02	.01	[illegible]
	CV	1 8	2.1	1 7	2.2	2.3	23
Area (μm²)	M	18	20	21	20	19	[illegible]
	SE	6	.8	.8	6	.5	[illegible]
	CV	3.5	4 1	3 6	3.1	2 4	33
Cytoplasm area (μm²)	M	24	31	33	37	44	[illegible]
	SE	2.5	2.8	1.3	1 4	1 1	12
	CV	10.2	8.8	4.1	3 7	2.6	[illegible]
Relative cytoplasm area %		50	66	68	77	92	[illegible]

time in the distribution of labelled RBC from class III to class VI must be interpreted as a result of maturation of RBC from class I to class VI. Further support for this are the results of the calculations of per cent labelled RBC per class of all RBC (Fig. 4). It can be seen in Fig. 4 that only small differences seem to exist in these curves from classes I–IV day 20 to 52. Thus, after 20 days these classes are constant in their composition with respect to the quotient between labelled and unlabelled RBC. Accordingly, the transit time (maturation time) for RBC from classes I–IV is shorter than 20 days. The level of labelled RBC in classes V and VI increases with time and days 41 and 52 have most likely the same distribution of labelled RBC. Thus the transit time for RBC from class I to class VI must be shorter than 41 days.



[illegible] 7. Incorporation of [59]Fe expressed as grain count per labelled RBC related to short/long axes ratio of [illegible] independent of the classification in Table I. Regression lines are given for the decreasing phase [illegible] ch observation time

[illegible]e projected RBC cytoplasmic area is used as a measure of the RBC volume the highest [illegible]centration of radioactive iron in the cells is found at all the sampling occasions in class [illegible] In this class the RBC has not achieved the final (mature) cell size. The radioactive iron [illegible] grains per projected RBC cytoplasmic area) in RBC from class VI is almost the same for [illegible] sampling days 20–52, with only a small but not significant increase.

The 6 classes represent a developmental series and the ratio between short and long axes [illegible] the RBC decreased from class I to class VI (Table II) and can be used as a measure of [illegible] degree of RBC maturation. Therefore the grain count per RBC was plotted against the [illegible]io between the short and long axes of the RBC (Fig. 7). The grain counts used are mean [illegible]lues calculated on RBC with equal ratios. As the grain counts are the same from 0.40–0.60 [illegible]nd then decrease with increasing ratio at days 15–41 the constants for the regression lines [illegible]e been calculated for the decreasing parts of the curves. The constants for the regression [illegible]nes are given in Fig. 7. The decreasing grain counts in classes III and IV from days 20–52 [illegible] Fig. 6 can be seen in Fig. 7 as decreasing slope for the regression lines.

Discussion

[illegible]ccording to Fowler *et al.* (1966) and Orlic (1968) [59]Fe gives a good resolution in auto-

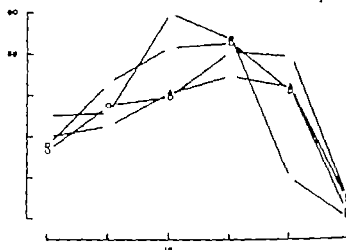


Fig. 5. Percentage of labelled RBC within each class I to VI (Table I) 15, 20, 41 and 52 days after the administration of a single dose of ^{59}Fe in each test fish

day 15 consists of RBC which have taken up a significant part of their iron before the radioactive form became available to the cells and therefore shows a low grain count and many cells are not above the background count.

As the projected area of the RBC cytoplasm is increasing from class I to class VI (Table I) the number of grains per projected area of cytoplasm was calculated. From Fig. 6 it can be seen that the increase in grain counts (the uptake of iron) is not proportional to the increase of the cytoplasm area. The highest grain counts are found in class IV day 20. It can also be seen that in classes III and IV the grain counts decrease from day 20 to day 52. This is most likely a result of decreasing specific radioactivity of ^{59}Fe in the blood with time

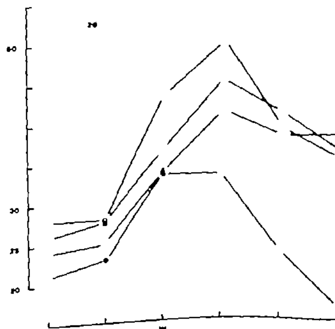


Fig. 6. Incorporation of ^{59}Fe in RBC belonging to each age class I to VI (Table I). The graphs represent the situation 15, 20, 41 and 52 days after the intraperitoneal injection of a single dose of the radionuclide. The incorporation is expressed as number of grain count per µm² of projected area (see Table II)

per RBC is achieved. This may explain the low detection of labelled RBC from the ...tages of RBC with low incorporation level (see Fig. 5). In later parts of the experi- ...l period the proportion of radioactive iron decreases and this will result in decreasing ...counts and a decreasing detection level of the RBC. This view is supported by the ...s that the highest per cent labelled RBC is found on day 15 when the ratio between ...ctive and nonradioactive iron can be expected to be at maximum in the present study. ...occurrence of ^{59}Fe in an early RBC as found in the present study does not necessarily ...that Hb-synthesis has started. Thus the iron detected by autoradiography may exist ...Hb, b) other ferroproteins, c) ferritin or transferrin inside the cell, and d) transferrin ...ed to the outer surface of the cell membrane. In mature RBC Hb is by far the ...nant ferroprotein. Hevesy *et al.* (1964) showed that ^{59}Fe incorporated into RBC from ...(*Tinca tinca*) ...than 24 h (at 18°C) was bound to the Hb with low amounts in the non- ...action. Walker and Fromm (1976) found that some iron accumulated by RBC in the ...ow trout ...as temporarily stored as non-Hb iron. These authors also discussed the ...nce of non-Hb bound iron in the cytoplasmic iron stores as a result of over-loading of RBC.

...e iron uptake by erythroid cells is not regulated by heme synthesis. Human developing ...have been shown to have an active uptake of iron from the ironbinding protein in the ...ma and then return the excess (see *e.g.* Harris and Kellermeyer 1972). The iron uptake ...ost likely regulated by the iron available to the cells (Harris and Kellermeyer 1972). ...s iron overloading of developing RBC may occur temporarily.

...he ...hite blood cells in the present study did not show grain counts exceeding the back ...and ...hich supports the view that the iron incorporated into ferroproteins (cytochromes, ...oxidases, and katalases) other than Hb can be neglected when the results in the present ...dy are discussed.

...Due to the fact that ^{59}Fe in the iron-binding proteins may be detected on the RBC mem- ...ne with higher efficiency than iron inside the cell and the possibility of non-Hb-bound ...n and overloading, correlation between ^{59}Fe uptake and the increase of Hb concentra- ...n in the RBC is restricted. Thus the labelling (measured as grain counts) cannot be used ...a quantitative measure of the RBC Hb-concentration. In young RBC with low Hb- ...ncentration the discrepancy between Hb and iron is greater than in mature RBC. This ...ll limit the estimations of the Hb-concentration in the different classes of RBC in the ...esent study.

The decreasing number of grains per projected cytoplasm area in class IV from day 20 ...day 52 (Fig. 6) and the decreasing slope of the regression lines for short/long axes ratio ...nd grain counts (Fig. 7) could indicate overloading of the cells in class IV. This might also ...e a result of decreasing specific radioactivity in the plasma.

...It can be concluded that Hb is synthesized in the RBC during maturation along the pro- ...posed series and it is not completed until the cells reach at least class IV short/long axes ...ratio 0.6.

No iron uptake was detected in RBC already mature at the time for the administration ...of the ^{59}Fe. This fact supports the theory that only the immature salmon RBC can take up ...iron as earlier has been found for mammalian RBC (*cf.* Harris and Kellermeyer 1972).

radiography presumably due to the comparatively low radiation energy of this material compared with Fe. Furthermore, this technique was chosen in the present study as active iron incorporated in human erythrocyte haemoglobin according to Gibson et al (19 gives rise to a permanent activity during the whole lifetime of the red cells. Hevesy et al (1964) and Weinberg *et al* (1976) have shown that Fe injected intraperitoneally in tench (*Tinca vulgaris*) and kissing gourami (*Heleostoma temmincki*) appears in liver, spleen, kidney and RBC Yu *et al* (1971) report that ^{59}Fe incorporated into erythrocytes of the gourami (*Trichogaster trichopterus*) nine days after the administration appeared as haemosiderin in the same organs. According to the same authors this source of iron was cycled during the erythropoiesis.

No reports have been found showing that fish erythrocytes are capable of cell division in the circulation. Thus it is assumed that RBC delivered to circulating blood only undergo maturational changes. Wintrobe (1974) presents a model of the production and kinetics of human leukocytes. In this model it is assumed that the cells move through the system in a more or less orderly manner as if in a pipeline. In the present study class VI represents mature RBC and thus classes I–V can be interpreted as the maturation compartment. The change with time of the maximum of labelled RBC from classes III to VI (see Table shown in Fig. 3 and the increase of labelled RBC in classes IV–VI with time presented in Fig. 4 must be interpreted as a *flow* of ageing RBC through the maturation compartment and classes I–V are interpreted as successive transformation stages in the RBC maturation compartment corresponding to the model of production and kinetics for human leukocytes presented by Wintrobe (*op cit*).

An estimation of the transit times for RBC through the maturation compartment from the increase of labelled RBC in class VI (the mature RBC) from day 20 to day gives a result of 14 days, and similar estimations for the transit time to class V gives a of 9 days. These transit times are only estimations since the sampling occasions have been chosen with too long intervals to permit exact calculations. No corrections have been made for differences in effectiveness in the labelling.

The autoradiographic technique used has certain limitations. Due to low radiation energy of ^{59}Fe, the grains observed in the emulsion mainly derive from desintegrations located in superficial parts of the RBC as radiation in the interior of the cells may be absorbed.

It was shown by Walker and Fromm (1976) that ^{59}Fe given intraperitoneally to rainbow trout (*Salmo gairdneri*) was adsorbed within 24 hours and equilibrium between the plasma ^{59}Fe pool and the tissue pool was attained 8 days after the i p injection The half time of the plasma iron in tench (*Tinca tinca*) has been determined to be 4 4 h by van Dijk (and 4 h by Hevesy *et al.* (1964) According to this rapid turnover the specific radioactivity of the iron pool of the plasma and the pools of the tissues will be in equilibrium. As flooding of the fish body iron stores was done in the present study by parenteral administration of large doses of iron, the injected ^{59}Fe was mixed with iron already present. The pool will then persist for a rather long time compared to the situation with flooded iron stores. As only a part of the iron incorporated into the developing RBC is radioactive and only the iron in the part of the RBC which is close to the emulsion is detected with the autoradiographic technique used in the present study great vari o in the detected

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Insulin-stimulated bile formation in cats

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Abstract

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effects of insulin on bile flow and composition were examined in fasting, chloralose-anesthetized cats lin in doses from 0.01 to 2.00 U/kg increased bile flow and biliary erythritol clearance without any ...able change in the difference between them, thus insulin presumably had no effect on ductular fluid sport. Continuous infusion of insulin (0.1 U/kg + 0.05 U/kg/min or 0.05 U/kg + 0.002 U/kg/min) ...ased biliary erythritol clearance by 22 %. The increase was caused by rise in the bile acid-independent ...tion of bile production and accompanied by parallel increase in the rates of biliary excretion of Na^+ Cl^- When ouabain, 80 μg/kg, was injected intraportally during insulin infusion the erythritol clearance, flow and the rates of biliary excretion of Na and Cl^- were lowered towards but not to their pre-...lin levels. The effects of insulin on these parameters were unchanged after atropine or gastrectomy and ...oxy-D-Glucose was without effect on bile production. The results indicate that administration of ...lin affects bile formation by stimulating the active transport of sodium across the canalicular mem-...ne.

...tz and Brooks (1963) first demonstrated the choleretic effect of insulin in dogs. This ...ect has been confirmed by others and also demonstrated in guinea pigs and humans (see ...nes 1976). However attempts to elucidate the mechanism of this choleretic effect have ...en conflicting results. In guinea pigs it was recently found that insulin acted primarily ...stimulating the secretion from the biliary ductal epithelium (Lundy Migliori and Jones ...77) ...hereas Jones (1976) found that insulin stimulated the canalicular bile production ...the dog with only minor effects on the ductular production of bile. I... the present experi-...ents the choleretic effect of insulin has been examined in cats and the results show that ...sulin stimulates the bile acid-independent fraction of bile production apparently by ...timulating the active transport of sodium across the canalicular membrane.

Methods

...asting cats (weight 3.2±0.1 (S.E.) kg), anesthetized with chloralose (50 mg/kg) and Nembutal (30 mg) ...from ... h, were used for the experiments. Catheters for infusion and blood sampling were placed in the ...femoral arteries and veins. Bile was collected from catheter placed in the choledocus after ligation of the

Thus cells belonging to the part of the developing RBC series which show low grain counts can be regarded as erythroid cells which synthesize Hb. The increase in Hb concentration cannot be correlated with increase in the detected iron. The Hb increase is most likely slower than the iron increase

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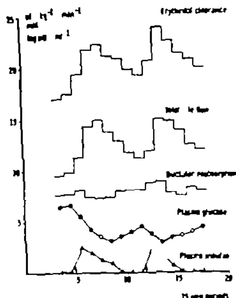
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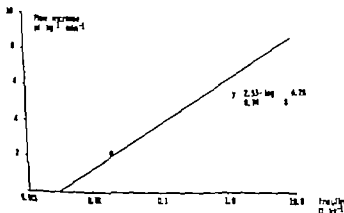
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The effects of injection of insulin 0.14 first arrow) and 0.07 U/kg (second arrow) on ... and the arterial plasma concentrations of ... and insulin

...usion of insulin. The effects of a priming dose of insulin (0.8 U/kg) i. followed by ...nuous infusion of insulin (0.05 U/kg/min) are listed in Table I and illustrated in Fig. 3 ...inistration of insulin resulted in an immediate rise in erythritol clearance which after ... 1 h stabilized at a level 22% above control flow for the rest of the experimental ...od. This was followed by parallel increase in the Na and Cl excretion rates. There ... a barely significant rise in the HCO_3 excretion rate whereas the K and bile acids excre... rates remained constant. It also appears that the ductular reabsorption of fluid cal... ...ted as erythritol clearance minus total bile flow was unaffected by the infusion of insulin. ...ving established that the maximum effects of insulin could be maintained for up to



...g. 2. The increase in total bile flow related to the amount of insulin injected.

cystic duct. To compensate for the abrupted enterohepatic circulation of bile acids taurocholate (... kg/min) was given i.v. Mean arterial blood pressure was recorded using a condenser manometer (... Schönander). Body temperature was maintained at 38.5°C by gentle heating.

Experimental design. In all expts. a period of 60 min was allowed for recovery from surgery ... arterial blood samples and bile were collected at 15-min intervals for 45 min. The following series ... were performed. a) 9 cats were given insulin (Insulin Leo® Neutral, Nordisk Insulinlaboratorium ... mark) in single doses i.v. ranging from 0.0015 U/kg to 2.0 U/kg, and the effects observed in ... periods. b) 5 cats were given a priming dose of insulin of 0.8 U/kg followed by continuous ... insulin 0.05 U/kg/min and the effects observed in about 10 15-min periods. c) 5 cats were ... as in series b. When the effect of insulin on bile flow had stabilized (after 75 min), ... the portal vein in single doses begin ing with 60 µg followed by 60 or 30 µg every 15 min until a ... of about 80 µg/kg was reached. d) In 5 cats insulin was given as a priming dose of 0.05 U/kg ... 0.002 U/kg/min and the effects followed for 10 15-min periods. e) In 4 cats ... alone was ... portally in the same doses as in series c. f) The effect of insulin (0.8 U/kg followed by 0.05 U/kg ... also tested in 2 cats with acute total gastrectomy and in cats in which atropine was given ... dose of 0.2 mg/kg i. followed by subcutaneous injection of 0.2 mg atropine 3 times during the ... mental period. g) Finally the choleretic effect of 2 Deo y-D-Glucose (Sigma) was tested in 3 ... were given 50–400 mg/kg i.v. as a single dose.

In all expts. plasma glucose and insulin concentration and bile flow and composition were ... The biliary clearance of ^{14}C-erythritol was determined by infusing 33 nC/min i. together with ... erythritol (2 µmol/min) after priming doses of 3 µC and 0.5 µmol, respectively.

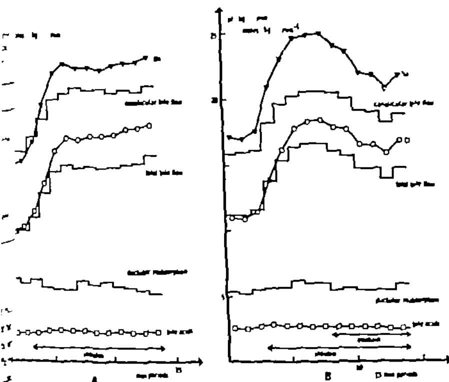
Analytical procedure. Bile flow was measured gravimetrically and the biliary concentrations ... acids, inorganic electrolytes and plasma glucose were measured as previously described (Kristensen and ... 1972). ^{14}C-activity in bile nd plasma specimens was measured in a Packard Tricarb liquid scintillation spectrometer and was corrected fo quenching by means of an external standard (Kristensen, Larsen, Munck 1976). Insulin was determined by a double-antibody technique using Wick chromatography ... Thomsen and Yde 1968).

Calculations. The biliary clearance of ^{14}C erythritol was calculated from the bile flow and the plasma ratio of C erythrit l. Assuming that this parameter reflects the canalicular bile production ... ler, Ross and Bradley 1968) the net eabsorption/secretion in the bile ductules was calculated as ... ference between erythritol clearance and bile flow. The biliary excretion of bile acids and ... was calculated as the product of bile flow and the biliary concentrations of the ions.

Statistical procedures. The effects of insulin on the flow and composition of the bile were tested ... Student's test for paired comparisons.

Results

Single injections of insulin. When insulin was injected i.v. in doses greater than 0.01 ... a rise in bile flow and a rise in ^{14}C erythritol clearance of similar magnitude was ... observed (Fig. 1). The increase in flow was manifest in the first sample after the inj... and peak bile flow was reached 2–4 samples after injection, depending on the amount ... insulin given. Also the time of return of flow to control values was dose-dependent ... insulin doses of 1–2 U/kg the plasma concentration of insulin rose above 500 µU/ml ... significant fall in plasma glucose concentration was observed as would be expected. ... bile flow was often reached before nadir glucose concentrations and was independent ... the initial plasma glucose level as well as the nadir plasma glucose concentration. When ... bile flow increase was related to the amount of insulin injected a correlation was found ... illustrated in Fig. 2. No obvious correlation between changes in bile flow and plasma ... sulin concentrations was observed. In these expts. the electrolyte excretion in bile was ... followed because of bile dead space interference.



... 5] The effect of insulin and insulin + ouabain on bile flow and composition. I part A insulin 0 8 ... 0 05 U/kg/min was given as indicated. In part B the same dose of insulin was given but in the last ... of the insulin infusion period ouabain was injected as indicated on the figure and described in the text

...ved in erythritol clearance or the fluid transport in bile ductuli. The composition of ... remained unchanged except for a rise in the biliary concentration of K^{+} and a corre... ding rise in the excretion rate of this ion from 0.53 ± 0.03 to 0.79 ± 0.05 10^{-} mol/kg/min ... 0 01) (Table II).

...LE II. Results from 4 expts. in which bile flow and the biliary excretion of Na^{+} and Cl^{-} were followed before and during injection of ouabain. The values are mean values ± S.E. of the last 3 samples in the control period and the last 4 samples in the ouabain period. Ouabain values are not statistically different from control values

	Control	Ouabain
...al bile flow (...l kg^{-1} min^{-1})	11.0 ± 0.8	12.2 ± 0.5
...ritol clearance (... kg^{-1} min^{-1})	14.7 ± 0.4	15.1 ± 0.9
...+ excretion rate (10^{-6} moles kg^{-1} min^{-1})	17.8 ± 0.1	18.6 ± 0.6
...⁻ excretion rate (10^{-6} moles kg^{-1} min^{-1})	12.2 ± 0.9	12.9 ± 0.6

TABLE I The effect of insulin (0 8 U/kg + 0.05 U/kg/min) on bile flow and composition The [illegible] mean values ± S E. from samples 1 3 (control), samples 6–8 (insulin I), and [illegible] (insulin II) from 5 expts. in which only insulin was infused (A) and from 5 expts in which [illegible] was injected during the last part of the insulin infusion period (B). Values from [illegible] compared with values from control and values from insulin II [illegible] values from [illegible]

	Control	Insulin I	Insulin II
Total bile flow (μl kg^{-1} min^{-1})	A 9 8 (1 4) B 11.3 (0 9)	15 0 (2.1) ** 16.4 (0.7)**	15 3 (2 2)[illegible] 14 4 (0 7)[illegible] [illegible]
Canalicular bile flow (μl kg^{-1} min)	A 16.2 (1.8) B 16 0 (1.4)	20.9 (2.4) 20 6 (1.2)	20 9 (2 9)[illegible] 18 7 (0 7)[illegible] + [illegible]
Na excretion rate (10^{-} moles kg^{-} min^{-})	A 15 1 ([illegible]) B 17.3 (1.3)	22.8 (3.2) 24 6 (1.3)*	[illegible] (3 9)[illegible] 21 4 (1 9)[illegible] [illegible]
Cl^{-} excretion rate (10^{-} moles kg^{-} min)	A 10 0 (2.1) B 11.2 (0.9)	17.2 (2.9) 18.2 (1 0)	18 1 (3 0)[illegible] 16 3 (1 0)[illegible] + [illegible]

ns = non-significant * = 0 01 < p < 0 05 ** = 0 005 < p < 0 01 *** = 0 001 < p < 0 005.

eight 15-min periods a second series of expts. was carried out in which the effects of ouabain on insulin-stimulated bile flow was examined. In this series insulin was administered continuously as described above. When the effect of insulin on bile flow had become stable (after five 15 min periods) ouabain was injected in the portal vein in single doses for the rest of the experimental period as described under Methods. The results are listed in Table I and illustrated in Fig. 3 It appears that injection of ouabain caused a significant reduction in erythritol clearance and also reduced the Na and Cl excretion rates. Ouabain caused a slight non-significant rise in K -excretion and a similar fall in HCO_3 -excretion rate, whereas the rate of excretion of bile acids remained constant When steady state flow was achieved the plasma concentration of insulin was about 8–12 mU/ml

The effect of insulin on bile flow and composition could also be produced by a priming dose of insulin of 0 05 U/kg followed by infusion of 0 002 U/kg/min In these expts the erythritol clearance increased from 14.5 ± 1 0 to 18 6 ± 1 1 μl/kg/min, and the rates of excretion of Na^+ and Cl increased from 16.5 ± 2.3 to 22.1 ± 2.3 and 10.8 ± 2.1 to 16 0 ± 2.3 [illegible] mol/kg/min, respectively Insulin caused a slight, but significant rise in HCO_3-excretion rate from 2.5 ± 0.2 to 3 5 ± 0.3 10 mol/kg/min and similarly a fall in K -excretion rate from 0 5 ± 0 02 to 0 4 ± 0.02 10^{-} mol/kg/min was observed. The rates of excretion of bile acids was not changed Once steady state flow was achieved the mean plasma insulin concentration was 105 ± 11 μU/ml

The plasma concentration of insulin in the control periods of the injection and the infusion expts. have been pooled together In the first plasma sample following the operative procedure insulin concentration varied considerably between 3–168 μU/ml with a mean value of 39 ± 11 μU/ml. 45 min later just before administration of insulin, the variation had declined to 2–72 μU/ml with a mean value of [illegible]1 ± 5 μU/ml

Injection of ouabain In 4 cats the effects of ouabain alone on bile flow and composition was tested Ouabain was given as single injections in the portal vein as described above and in similar doses. Ouabain did not affect total bile flow rate [illegible] er was any change

insulin-induced rise in erythritol clearance accompanied by a marked, parallel increase and Cl⁻ excretion rates and a small increase in HCO excretion indicate that insulin [illegible]ulate a Na K -ATPase in bile canaliculi. If this was the case, ouabain would be [expect]ed to abolish these effects of insulin. The limited effect of ouabain neither proves [nor dis]proves the validity of this concept. The fact that we found only partial depression [of] insulin-induced increments in bile flow and Na -excretion may have been due to [inadequ]ate doses of ouabain this could also explain the lack of effect of ouabain on bile [i]n the control expts. However attempts to increase ouabain doses beyond 80 μg/kg [prob]ably resulted in sudden cardiac failure.

[Wh]ether insulin influences bile secretion directly or indirectly cannot be answered con[clusiv]ely but several ways of action may be excluded. Neither total gastrectomy which [inclu]des truncal vagotomy nor injection of atropine interfered with insulin-induced choleresis [in th]e cat. The vagus is thus excluded as a possible mediator of the insulin effect. The lack [of eff]ect of gastrectomy on the choleretic action of insulin also excludes gastrin as a mediator [of th]e insulin effect. The constancy of the ductular fluid transport, as well as the lack of [chan]ge of biliary excretion of HCO during insulin administration makes it very unlikely [that] secretin should be responsible for the effect of insulin in the cat. Glucagon has been [sugg]ested as a possible mediator of the insulin effect, but previous expts. on cats (Krarup [&] Larsen 1974) demonstrated that glucagon, even when infused in high doses, gives a [chara]cteristic delayed choleretic response. cAMP a second messenger coupled to the [actio]n of glucagon and its dibutyryl derivative were without effect on bile flow (Krarup *et al.* [197]1). Prostaglandins E_1 and E_2 are potent choleretics in the cat (Krarup *et al.* 1976), but [they] exhibit a secretion-like effect without influencing canalicular bile production. It is well [kno]wn that hypoglycemia is accompanied by increased production of cathecholamines but [adren]alin and noradrenalin do not influence bile flow in cats (Krarup 1973).

Hypoglycemia, as such, has been suggested to evoke an increase in bile flow but in the [pre]s[ent] [w]ith single injections of insulin there was no correlation between insulin-induced bile [flow] and plasma glucose concentration. In contrast, the results from the infusion expts. [sho]w that bile flow and plasma glucose concentration are closely related. These results [ther]efore suggest that hypoglycemia could be directly responsible for the increase in bile [flo]w However in [a]greement with the expts. of Konturek, Kieta-Fyda and Moczurad (1967) [in] cats, but contrary to findings in dogs (Jones *et al.* 1970, Geist, Jones and Hall 1970, and [Ba]rnley and Jones 1972), the cytoglucopenic agent, 2 Deoxy D-Glucose, did not effect bile [flo]w [w]hich seems to speak against a direct effect of hypoglycemia.

The question then arises whether the choleretic effect might be caused directly by insulin. [Th]e main condition for [a] direct action of insulin seems to be fulfilled, namely that insulin [sh]ould come into contact with the ATPase sites in the bile canaliculi membrane. It is well [es]tablished that insulin is inactivated by the liver but according to the work of Lopez-[Q]uijada and Goñi (1967) and Daniel and Henderson (1970) insulin can pass the liver cell [in] apparently undamaged form in a variety of species including the cat, and insulin has [be]en found in bile in the same concentration as found in portal venous blood. It was further [m]ore calculated that the time required for insulin to pass through the liver cell to the bile [w]as about 17 min. Based on this information it would be expected that rather than being

Gastrectomy and administration of atropine or 2 Deoxy D-Glucose For further elucidation of the mechanism of action of insulin on bile flow a series of experiments was carried out in which the effect of acute gastrectomy or administration of atropine (see methods) were tested. Two cats were gastrectomized and following the usual recovery and control period insulin was given as a single dose of 0.8 U/kg supplemented by 0.05 U/kg per hr. Bile flow and composition in the control period appeared to be unaffected by gastrectomy and the infusion of insulin was accompanied by the choleretic response normally observed. In other cats it was found that administration of atropine in doses which are supposed to block all vagal reflexes, did not alter the choleretic effect of insulin. Finally 3 cats were given 2 Deoxy D-Glucose as single doses ranging from 50 to 400 mg/kg. It appeared that this cytoglucopenic agent did not alter bile flow or the electrolyte composition of bile in any way

Discussion

The present experiments have shown that injection of insulin stimulates bile flow also in the cat The effect of graded doses of insulin on bile flow is in accordance with the expts. of Jones, Geist and Hall (1970) on conscious dogs and an increase in flow rate of about 5 μl/kg/min at an insulin dose of 1–2 U/kg is compatible with the results of these and other expts. (*e g* Geist and Jones 1971 a, b, Gourlay and Jones 1972). This indicates that the experimental procedures employed including anesthesia, is without major importance for this effect of insulin. According to Jones *et al* (1970) the effect of insulin reaches a maximum at a dose of 1 U/kg. This saturation phenomenon is also observed in the expts. with continuous infusion of insulin in which a 20 fold increase in the amount of insulin given, and a corresponding rise in plasma insulin concentration from about 100 μU/ml to 1-1 U/ml, was without significant influence on the increase in bile flow Thus the effect of insulin on bile flow is only dose-dependent within certain narrow limits. This may explain why no correlation between the rise in bile flow and the plasma insulin and glucose concentrations was found in the single injection expts.

The expts. with continuous infusion of insulin reveal that the effect of insulin on bile flow can be maintained for hours. Judged from erythritol clearance, insulin increased the canalicular production of bile flow while the calculated ductular net fluid transport appeared to be constant. This is in accordance with the findings of Jones (1976) on dogs but is in contrast to the results of Lundy *et al* (1973) with guinea pigs, where insulin stimulated the ductular portion of bile production. Jones (1976) found a small increase in the biliary excretion rate of bile acids but in our expts. the excretion rate of bile acids was unaffected This means that insulin infusion selectively stimulates the bile acid-independent fraction of canalicular bile production This portion of bile production is claimed to be dependent on the active transport of inorganic ions and the inhibitory effect of ouabain on this bile production (Erlinger *et al* 1970 Boyer 1971) suggests that the active transport is due to a Na–K ATPase. This is supported by the existence of Na^+–K ATPase activity in bile canaliculi enriched plasma membranes from rat liver (see Reichen and Paumgartner 1977), and expts. in progress in our laboratory have also shown Na –K ATPase activity in isolated bile canaliculi from the cat.

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correlated to arterial plasma insulin concentrations, bile flow should be correlated to the insulin concentrations in bile or portal venous blood. However these measurements have not been made.

It would be of interest to know whether insulin affects the activity of the Na-K-ATPase system from the bile canaliculi "in vitro" but no information is available on this point. However in expts. with toad bladder the results indicated that insulin stimulates the active sodium transport across the membrane by unmasking latent pump sites and by [illegible] formation of new pump sites (see Wiesmann, Sinha and Klahr 1977). If this mode of action is applied to the present results it may explain the difference in sensitivity of bile production to ouabain in the control and insulin expts.

In conclusion, the present expts. have demonstrated that in the cat administration of insulin stimulates the bile acid-independent fraction of canalicular bile production most probably by stimulating the active transport of sodium across the canalicular membrane. Whether or not this effect is caused by a direct action of insulin remains to be shown, but the results suggest that this may be the case.

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rate. The results indicated that energy flux decreased somewhat during the first 30 min, ing i.p. administration of amphetamine in a dose of 5 mg kg^{-1} but that there was a quent increase by 25–30%. Furthermore, two groups of workers (Naborski and 1975 King et al. 1975) have observed that amphetamine administration leads to an se in tissue concentrations of malate and, following the suggestion of Goldberg et al.), this was assumed to reflect an increased metabolic rate.

cently we reported in preliminary communications that amphetamine sulphate in doses (5–15 mg kg^{-1}) induced a marked increase in CBF and CMR$_{O_2}$ in paralyzed Carlsson et al. 1975, Berntman et al. 1976). Under some circumstances, the increase in was so pronounced that it became a matter of speculation whether the method used of sufficient accuracy to allow reliable derivation of CMR$_{O_2}$. We therefore undertook a er modification of the technique, designed to increase accuracy of the estimation of and CMR$_{O_2}$ in high flow situations (Berntman et al. 1978).

the present experiments, we have repeated and extended the previous studies on cir tory and metabolic effects of amphetamine in paralyzed and artificially ventilated rats, ded measurements of regional CBF (rCBF) and performed extensive measurements of e tissue metabolites. It will be shown that amphetamine markedly increases CBF cially in frontoparietal cortical regions, but that the effects on CMR$_{O_2}$ are less than iously reported. Apart from causing glycogenolysis, amphetamine has small effects on e metabolites but, after large doses and long exposure periods, significant changes are erved in some glycolytic metabolites and citric acid cycle intermediates.

Methods

experiments were performed on male Wistar rats, weighing 305–395 g. Animals ere housed in num- of 4 in plastic cages, and had free access to pellet food and water. Experiments were performed between a. and 3 p.m.

ration and sampling techniques

anaesthetized animals were used for studies of clinical behaviour following i.p. injection of d,l-amphet- ine sulphate in doses of 5 or 15 mg kg^{-1}. Other animals were provided ith tail artery catheters under lothane (2 3) anaesthesia (see Ponten and Siesjö 1967, Lewis et al. 1973). About 2 h later hen the mals had recovered from the anaesthesia, arterial samples were collected before and after i.p. injection amphetamine (5 mg kg^{-1}) for measurements of Pa_{CO_2}, P_{O_2}, and pH.

All other expts. were conducted on animals that were anaesthetized ith 2–3 % halothane (operative prepara- tion) and then maintained on 70 % N_2O and 30 % O_2 without any halothane. Immobilization as achieved th subocurarine chloride (1 mg kg^{-1}), and artificial ventilation was adjusted to give arterial CO_2 ten- sion of 35–40 mmHg. Body temperature was adjusted close to 37°C.

In animals used for studies of CBF and CMR$_{O_2}$, two femoral arteries and one femoral vein were can- nulated, and the posterior part of the superior sagittal sinus was exposed for sampling of cerebral venous blood. When rCBF was studied, no burr hole as placed, and one of the arterial catheters were cut to ength of about 2 cm (to minimize smearing). Preparations for studies of labile cerebral metabolites in- cluded placing skin cement over the intact skull bone for later in situ freezing according to Ponten et al. 1973). In many of these animals, the atlantooccipital membrane was exposed for sampling of cisternal CSF ith sharp glass pipette.

Methods for CBF and CMR$_{O_2}$

Cerebral blood flow (CBF) was measured in two series of animals (A and B). Series A was studied in the fall of 1975 and spring of 1976 and the results have been summarized in two preliminary communications

Acta physiol. scand. 1978. 102. 310–323

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Circulatory and metabolic effects in the brain induced by amphetamine sulphate

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Abstract

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Cerebral circulatory and metabolic effects of amphetamine sulphate (0.25–25 mg kg⁻¹ i or 5–30 mi i.p.) were studied in anesthetized, paralyzed and artificially ventilated rats. Cerebral blood flow (CBF) was measured with a modification of the Kety and Schmidt (1948) technique, and oxygen consu (CMRo$_2$) was calculated from CBF and arteriovenous differences in oxygen content. Regional CBF evaluated from the uptake of ^{14}C-ethanol. Cortical metabolites were analyzed following freezing of *in situ* Amphetamine administration gave rise to a marked increase in CBF that as doubled fol 0.25 mg kg⁻¹ and increased 4-fold following 15 mg kg^{-1} However such excessive increases in flow confined to frontoparietal cortical regions, while other cortical or subcortical areas showed more mo hyperemia The increase in CBF was unrelated to changes in arterial Pco$_2$, blood pressure, or tissue content CMRo$_2$ increased by 30% to 95% depending on dose and rat strain used At all doses amphetamine gave rise to glycogenolysis in cerebral cortex but in animals studied within the first after 5 mg kg^{-1} or less, the only other changes were increases in glucose-6-phosphate and α-lactate concentrations. When the dose was increased to 15 mg kg⁻¹ there were moderate increases in lactate concentration and lactate/pyruvate ratio. Sixty min after 5 mg kg⁻¹ there were increases in tissue concentrations of pyruvate, citric acid cycle intermediates and alanine, as well.

It has been well established that administration of amphetamine to rats and mice in locomotor activity and induces a stereotyped behaviour (see symposium edited by and Garattini 1970) Biochemical and pharmacological studies indicate that the effe due to release of catecholamines from central monoaminergic neurons (*e.g.* Carlsso 1970). Several studies have shown that amphetamine leads to glycogenolysis in the (Estler and Ammon 1967 Hutchins and Rogers 1970, 1971 Rogers and Hutchin Nahorski and Rogers 1973 however see also King *et al.* 1975) probably by mech involving conversion of phosphorylase *b* to *a* (Nahorski and Rogers 1974). There i indirect evidence that amphetamine increases metabolic rate in the brain. Nahors Rogers (1973) applied the "closed system" method of Lowry *et* to estimate

…ose, lactate and pyruvate were measured in arterial blood and CSF as well. Intracellular glucose …trations were calculated by assuming that blood and extracellular fluid occupy 3 and 15 …, re…ly of tissue volume, and that CSF and extracellular glucose concentrations are equal.

…cal significance of differences in means … ere determined by comparing control values with ex…ntal values, using Student's t-test. … p 0.01 p 0.001 …

Results

…sults will be presented under the following headings. (1) behavioural effects, (2) effects …ysiological variables, (3) glucose distribution, (4) tissue metabolites, and (5) CBF and …. Tissue metabolites (and glucose distribution) were only measured in series A while …her variables were studied in both series A and B (see Methods).

…havioural effects

…anesthetized animals of both series were injected i.p. with amphetamine in doses of 5 or … kg⁻ and their behaviour was observed for at least 60 min. All animals showed …ges in behaviour previously reported by others, i.e. increased mobility, piloerection, …thalmus, salivation and stereotyped gnawing and rearing. The symptoms were usually …ceable within 5–10 min, the stereotyped behaviour being most marked following 15 … kg^{-1}

…Effects on physiological variables

…e animals of series A and 2 of series B were provided with tail artery catheters under …othane anesthesia. About 2 h later, when the animals had fully recovered, they were …cted i.p. with 5 mg kg^{-1} of amphetamine. Arterial samples were collected every 15 min … measurements of $P_{a_{O_2}}$, P_{CO_2} and pH. There were no consistent variations in P_{O_2}. Changes … $P_{a_{CO_2}}$ and pH are shown in Fig. 2. In animals of series A, mean Pa_{CO_2} fell to about 27 mmHg …ing the first 15 min, and values remained below 30 mmHg during the 60 min observation …iod. Arterial pH varied in accordance with the fall in P_{CO_2}. The two animals of series B …owed a less pronounced hyperventilation, values remaining between 30 and 35 mmHg …ring the 45 min observation period.

Previous results have shown that administration of amphetamine to spontaneously …athing animals leads to hyperthermia (see Lewander 1971, Nahorski and Rogers 1976). …ce both hyperthermia and hypocapnia can, by themselves, induce changes in cerebral …ood flow and metabolism, the ventilation of the present anesthetized animals was …djusted … to maintain P_{CO_2} at 35–40 mmHg, and body temperature was kept close to 37°C (since … O_2 was given, arterial P_{O_2} was above 100 mmHg in all animals). Our main concern …re therefore changes in blood pressure and pH as well as in lactate and pyruvate concentra…ons.

Rats under 70% N_2O usually have *blood pressures* around 150 mmHg. Following i.v. …jection of amphetamine in doses of 0.25–25 mg kg^{-1} there was invariably an increase in …lood pressure of 10–25 mmHg, an example of which is given in Fig. 3. However, this in-

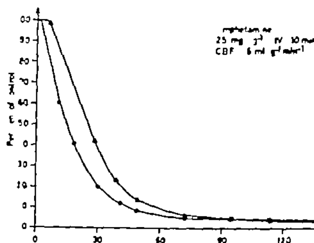


Fig. 1 Representative ^{133}Xe desat... curves obtained after intravenous inj... amphetamine (25 mg·kg^{-1}) Circles ... arterial samples and triangles venous ... Time in sec.

(Carlsson *et al.* 1975, Berntman *et al.* 1976). Series B was studied after July 1976. In the meantime ... supplier of animals bred a new strain of Wistar SPF rats. In both series, the basic methodology was the ... (Kety and Schmidt 1948) and involved saturation of animals with ^{133}xenon, and repeated measurements of ^{133}xenon activity in arterial and cerebral venous blood during desaturation (Eklöf *et al.* 1973, Norberg and Siesjö 1974). However in series A a 5 sec lag period was allowed, following start of desaturation, before first blood samples were collected, and it was seldom possible to collect more than 3 blood samples during the first minute of desaturation. This method has previously been used to measure CBF (and CMRo$_2$) in two other high flow situations, *viz* hypercapnia and hypoxia (Eklöf *et al.* 1973, Johannsson and ... 1974). Since accuracy at these high flow rates was considered unsatisfactory the method was further modified with omission of the 5 s lag period and more frequent sampling of arterial and cerebral venous blood during desaturation (Berntman *et al.* 1978). This modification was used on animals of series B. Fig. 1 ... that, with this technique, it was possible to accurately assess the arterial and venous desaturation ... even when CBF exceeds 5 ml g^{-1} min^{-1}. From curves obtained in normoxia, hypoxia and hyperca... could be estimated that the previous technique led to an overestimation of CBF by about 5% at ... flows, and by about 15% at increased flows. These correction factors were used to recalculate previously published values (series A).

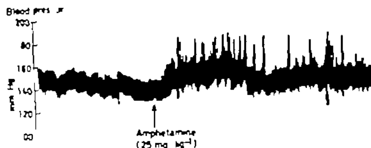
Arterial and cerebral venous samples for measurements of oxygen content (Co_2) were obtained just prior to desaturation, and about 1 min after start of desaturation. If arteriovenous differences in oxygen content ($AVDo_2$) did not differ by more than 10%, the mean value was multiplied with the calculated CBF to give CMRo$_2$. If the difference was larger the experiment was discarded.

Regional CBF (rCBF) was measured with the technique of Landau *et al.* (1955 see also Reivich ... 1968), as applied to ^{14}C-ethanol (Eklöf *et al.* 1974). Infusion time was 30 s. Brain regions were dissected at -10°C before weighing.

Analytical techniques

Arterial Po_2, Pco_2 and pH were measured with microelectrodes at 37°C, and corrections were applied for any difference in temperature between electrodes and animals. Arterial and cerebral venous oxygen contents were measured on 25 µl samples, using the polarographic method of Fabel and Lübbers (1964, see ... ström *et al.* 1974).

For studies of *metabolite concentrations* in blood and CSF samples were collected in liquid ... Following freezing of brain tissue *in situ*, pieces of frontoparietal cortex were dissected at -22 ... blood, CSF and tissue samples were extracted at -22°C with HCl-methanol, further extraction ... perchloric acid was carried out at 0°C, and the neutralized extracts were subsequently analysed for ... bolites, using the enzymatic, fluorometric techniques of Lowry and Passonneau (1972), ... conditions as given previously (Folbergrová *et al.* 1972a and b, 1974a and b). The following metabolites were measured (in tissue) phosphocreatine (PCr), creatine (Cr), ATP ADP AMP glycogen, glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), fructose-1,6-diphosphate (FDP), dihydroxyacetone phosphate (DHAP), 3-phosphoglycerate (3-PG), pyruvate lactate, citrate α-ketoglutarate (... fumarate, malate, glutamate, aspartate, glutamine, GABA, alanine and a...



Typical blood pressure recording showing effects of intravenous injection of amphetamine (25 mg ... 60 min period.

...tion was somewhat more pronounced, and there was a significant rise in lactate/...ate ratio. However at the end of 30 min, there was no statistically significant difference ...od lactate concentrations although the lactate/pyruvate ratio did differ (p 0.05). CSF ...e and pyruvate concentrations were similar in control and amphetamine-injected ...s.

...lucose distribution

...ose concentrations in blood, CSF and tissue were measured 30 and 60 min after 5 ...g⁻¹ of amphetamine, as well as 30 min following 15 mg kg⁻ (Table II). Intracellular ...ntrations were calculated on the assumption that blood and extracellular fluids oc... 3 and 15%, respectively of tissue volume. In general amphetamine had little effect on ...d and CSF glucose concentrations. However there were consistent (but not significant) ...ses in intracellular to blood concentration ratios following 5 mg kg⁻ and a significant ... after 15 mg kg⁻ Clearly the experiments failed to show that amphetamine reduces ...e to blood glucose concentration ratios.

... Tissue metabolites

...n tissue was frozen *in situ* for metabolite analyses following amphetamine in doses of ...mg kg⁻¹ *i.v.* (30 sec, 2 min and 5 min), 5 mg kg⁻ *i.p.* (30 and 60 min) and 15 mg kg⁻¹ ... (30 min). Results on cerebral energy state were consistent since no group of animals in...ted with amphetamine showed any variation from control in tissue concentrations of ..., Cr ATP ADP or AMP Cyclic AMP concentrations were measured 60 min after ...mg kg⁻¹ The values (1.81 ± 0.04 μmol g⁻¹) did not differ from those of the controls ...74 0.06 μmol g⁻¹).

In animals that were frozen within the first 30 min following injection of amphetamine in ...ses of 2.5–5 mg kg⁻¹ metabolic changes were relatively slight. Table III shows results ...tained with 2.5 mg kg⁻¹ *i.v.* (5 min), 5 mg kg⁻¹ *i.p.* (30 min) and 15 mg kg⁻¹ *i.p.* (30 min). ...tissues of animals from the last group were extracted against their own controls. In the table, ...e two control groups were pooled but statistical differences were calculated against the ...pooled control groups. Consistently there was fall in glycogen concentration, and in...ses in G-6-P and α-KG concentrations. In animals given 2.5–5 mg kg⁻ of amphetamine

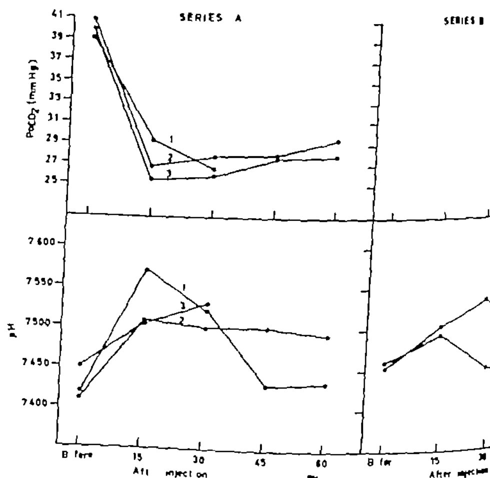
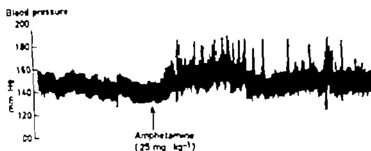


Fig. 2. Changes in arterial P_{CO_2} and pH in series A and B after intraperitoneal injection of amphetamine in spontaneously breathing rats.

crease was relatively short-lived and at 15, 30 or 60 min following i.v. or i.p. administration of amphetamine in doses of 2.5–15 mg kg⁻ blood pressure was close to control. Thus, the increases in CBF to be described below were unrelated to changes in pressure (or P_{CO_2}).

Temperature, mean arterial blood pressure, arterial P_{O_2}, P_{CO_2} and pH were measured in all groups (series A and B) used for measurements of CBF and CMR_{O_2} and in those used for tissue metabolite analysis. All values were very similar to those of the control groups, except that both series showed a plasma acidosis with time, even though ventilation was controlled and Pa_{CO_2} remained close to 40 mmHg.

Lactate and pyruvate concentrations were measured in blood and CSF 60 min after 5 mg kg⁻ of amphetamine, as well as 15 (blood) and 30 (blood and CSF) min following 15 mg kg⁻. Table I shows that following 5 mg kg⁻ of amphetamine, there was a slight increase in blood lactate concentration and lactate/pyruvate ratio. The mean CSF concentration of lactate was somewhat higher than in controls, but the difference was not statistically significant. Following 15 mg kg⁻ blood lactate concentrations in all animals rose somewhat between 15 and 30 min in all animals but there was no increase



Typical blood pressure recording showing effects of intravenous injection of amphetamine (25 mg kg⁻¹) 60 min period.

...ion was somewhat more pronounced, and there was a significant rise in lactate/...te ratio. However at the end of 30 min, there was no statistically significant difference ...d lactate concentrations although the lactate/pyruvate ratio did differ ($p < 0.05$). CSF ...and pyruvate concentrations were similar in control and amphetamine-injected

...cose distribution

...se concentrations in blood, CSF and tissue were measured 30 and 60 min after 5 ...$^{-1}$ of amphetamine, as well as 30 min following 15 mg kg⁻ (Table II). Intracellular ...trations were calculated on the assumption that blood and extracellular fluids oc... 3 and 15%, respectively of tissue volume. In general, amphetamine had little effect on ...d and CSF glucose concentrations. However there were consistent (but not significant) ...ses in intracellular to blood concentration ratios following 5 mg kg⁻ and significant ...after 15 mg kg⁻ Clearly the experiments failed to show that amphetamine reduces ...e to blood glucose concentration ratios.

Tissue metabolites

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TABLE I Lactate and pyruvate in arterial blood and CSF (in µmol g⁻¹) after i.p. administration of [illegible] mine compared to control groups. N O anesthesia. n = number of animals. Mean ± [illegible]

Groups	n	Blood Lactate 15 min	Blood Pyruvate 15 min	Blood La/Py 15 min	Blood Lactate 30 min	Blood Pyruvate 30 min	Blood La/Py 30 min	CSF Lactate	CSF Pyruvate	[illegible]
Series A										
Control	6	2.17 ±0.20	0.197 ±0.005	11.0 ±0.8	2.83 ±0.28	0.211 ±0.015	14.3 ±2.7	2.49 ±0.08	[illegible]	[illegible]
Amphetamine 15 mg kg⁻¹	6	2.08 ±0.27	0.187 ±0.019	11.1 ±0.9	3.46 ±0.63	0.179 ±0.017	19.3* ±1.5	2.18 ±0.21	[illegible]	[illegible]
					60 min			60 min		
Series B										
Control	6				1.42 ±0.26	0.154 ±0.021	9.2 ±1.1	2.62 ±0.10	[illegible]	[illegible]
Amphetamine 5 mg kg⁻¹	6				3.14 ±0.64	0.167 ±0.011	17.4* ±2.9	2.44 ±0.17	[illegible]	[illegible]

the only other changes were reduction in DHAP (2.5 mg kg⁻¹ i.v. 5 min) or 3-PG (5 mg kg⁻¹ i.p., 30 min) concentrations. In spite of the fact that only some of the differences were statistically significant glycolytic changes appeared consistent since the mean values for G-6-P and F-6-P were increased and those for FDP DHAP GAP and 3-PG decreased. However pyruvate and lactate concentrations remained constant unless the dose of amphetamine was increased to 15 mg kg⁻¹ In the latter group, both the lactate concentration and lactate/pyruvate ratio were slightly elevated

In animals frozen 60 min after 5 mg kg⁻¹ of amphetamine, metabolic changes were [illegible]

TABLE II Glucose concentrations (µmol g⁻¹) in arterial blood, CSF cortical tissue and calculated concentration in intracellular water (ICW), as well as ratios between ICW and blood, after amphetamine injection (i.p.) compared to control groups. n = number of animals.

Groups	n	Glucose Blood	Glucose CSF	Glucose Tissue	Glucose ICW	ICW [illegible]
Series A						
Control	6	12.6. ±1.07	5.32 ±0.23	4.32 ±0.32	5.15 ±0.45	0.41 ±0.02
Amphetamine 15 mg kg⁻¹ 30 min	6	10.00 ±0.77	5.65 ±0.33	4.09 ±0.28	5.06 ±0.37	[illegible] ±0.02
Series B						
Control	6	10.32 ±0.46	5.95 ±0.16	3.84 ±0.19	4.31 ±0.28	0.41 ±0.02
Amphetamine 5 mg kg⁻¹ 30 min	6	9.27 ±0.64	5.03 ±0.15	3.65 ±0.25	4.28 ±0.36	0.46 ±0.02
Amphetamine 5 mg kg⁻¹ 60 min	6	11.05 ±0.84	5.43 ±0.29	4.34 ±0.40	5.24 ±0.57	0.47 ±0.02

The concentrations (mean ± S.E.) of glycolytic, citric acid cycle metabolites and some associated amino acids in cerebral cortex after amphetamine administration. Values are given in µmol g⁻¹ … number of animals.

	Control	Amphetamine		
	12	2.5 mg kg⁻¹ i., 5 min, 3	5 mg kg⁻¹ i.p., 30 min, = 6	15 mg kg⁻¹ i.p., 30 min, = 6
	2.29 ± 0.15	2.07 ± 0.13	1.43 ± 0.10***	1.40 ± 0.08*
	4.08 ± 0.24	4.15 ± 0.21	3.65 ± 0.25	4.09 ± 0.28
	0.077 ± 0.004	0.095 ± 0.006	0.084 ± 0.003	0.103 ± 0.006
	0.011 ± 0.001	0.012 ± 0.001	0.012 ± 0.001	
	0.121 ± 0.002	0.115 ± 0.003	0.114 ± 0.002	
	0.024 ± 0.001	0.021 ± 0.001	0.022 ± 0.001	
	0.035 ± 0.001	0.032 ± 0.004	0.029 ± 0.002*	
	0.147 ± 0.006	0.158 ± 0.008	0.151 ± 0.006	0.151 ± 0.006
	1.83 ± 0.06	1.83 ± 0.14	1.73 ± 0.07	2.34 ± 0.16
	12.6 ± 0.3	11.1 ± 0.5	11.4 ± 0.3	15.9 ± 0.5*
	0.134 ± 0.006	0.173 ± 0.012	0.169 ± 0.007*	0.192 ± 0.012*
	0.069 ± 0.003	0.070 ± 0.002	0.072 ± 0.002	
	0.410 ± 0.016	0.411 ± 0.025	0.419 ± 0.013	0.445 ± 0.032
	12.82 ± 0.32	13.16 ± 0.11	13.11 ± 0.14	
	3.40 ± 0.11	3.33 ± 0.12	3.29 ± 0.02	
	5.95 ± 0.10	5.78 ± 0.27	6.26 ± 0.27	
	1.94 ± 0.05	2.02 ± 0.04	2.02 ± 0.04	
	0.536 ± 0.018	0.509 ± 0.006	0.517 ± 0.014	0.592 ± 0.024
	0.272 ± 0.023	0.252 ± 0.018	0.229 ± 0.018	0.282 ± 0.029

…ounced (Table IV). Thus, there were significant increases in the concentrations of …te and pyruvate, of all citric acid cycle intermediates except α-KG and of alanine, as … a small decrease in GABA concentration.

…BF and CMR_{O_2}

…ts on CBF and CMR_{O_2} were not consistent between series A and B. In series A, our …minary expts. indicated that amphetamine in doses of 2.5–5 mg kg⁻¹ gave rise to a …-fold increase in CBF and to a 30–40% increase in CMR_{O_2} within the first 30 min, … further increases 60 min after 5 mg kg⁻¹ or 30 min after 15 mg kg⁻¹ (Carlsson *et al* …, Berntman *et al* 1976). As Table V shows, there were differences in results between …s A and B, especially in animals studied 30 min after 15 mg kg⁻¹ or 60 min after 5 … kg⁻¹ These differences may reflect a difference in response to amphetamine between …als (see Discussion). As judged from the (more precise) measurements obtained in …s B, CMR_{O_2} increases by about 25% following amphetamine in doses of 2.5–5 mg kg⁻¹ …rthermore, the results provide some suggestion that the effects on CBF are dose-dependent … the range 0.25–25 mg kg⁻¹ With the largest dose, the CBF values obtained suggest that …phetamine may induce "maximal" vasodilatation in the brain (see Discussion).

In order to validate the marked increase in CBF estimated by the ¹³³xenon technique, and … obtain information of CBF distribution, regional CBF (rCBF) was evaluated from the …ne uptake of ¹⁴C-ethanol (see Methods). Since this technique somewhat underestimates …BF especially at high flows, rCBF was also measured in hypercapnia. As Table VI shows,

TABLE IV Glycolytic metabolites, citric acid cycle intermediates, associated amino acids and [illegible] 60 min after amphetamine injection, compared to a control group. Values in mmol g^{-1} [illegible] S.E. n = number of animals.

	Control n = 6	Amphetamine 5 mg kg^{-1} i.p., 60 min n = 6
glycogen	[illegible]4 ± 0.22	1.[illegible] ± 0.08
glucose	3.80 ± 0.[illegible]1	4.34 ± 0.41
G-6-P	0.102 ± 0.007	0.111 ± 0.009
F-6-P	0.014 ± 0.004	0.016 ± 0.001
FDP	0.078 ± 0.005	0.070 ± 0.007
DHAP	0.039 ± 0.003	0.039 ± 0.002
3-PG	0.034 ± 0.003	0.034 ± 0.003
pyruvate	0.121 ± 0.006	0.138 ± 0.004
lactate	1.54 ± 0.09	1.96 ± 0.09
la/py	12.6 ± 0.4	14.2 ± 0.6
α-KG	0.153 ± 0.009	0.176 ± 0.013
fumarate	0.065 ± 0.004	0.076 ± 0.003
malate	0.343 ± 0.021	0.407 ± 0.010*
glutamate	12.88 ± 0.13	13.31 ± 0.15
aspartate	3.37 ± 0.07	3.25 ± 0.05
glutamine	5.67 ± 0.18	5.43 ± 0.23
GABA	2.58 ± 0.05	[illegible].38 ± 0.05
alanine	0.457 ± 0.020	0.518 ± 0.015
ammonia	0.326 ± 0.021	0.331 ± 0.011

TABLE V Total arterial oxygen content (Ta_{O_2}), arteriovenous difference for oxygen (AVD_{O_2}), cerebral blood flow (CBF) and cerebral metabolic rate for oxygen (CMR_{O_2}) in rats after administration of amphetamine in two series compared to control groups (means ± S.E.). n = number of animals.

Groups	n	T_{O_2} ml 100 ml^{-1}	AVD_{O_2} ml 100 ml^{-1}	CBF ml 100 g^{-1} min^{-1}	CMR_{O_2} ml 100 g^{-1} [illegible]
Series A					
Control	5	23.89 ± 0.46	8.53 ± 0.6[illegible]	115 ± 8	10.0 ± 0.2
Amphetamine					
2.5 mg kg^{-1} i.v. 10 min	6	23.09 ± 0.69	3.31 ± 0.59*	453 ± 85*	15.0 ± 1.7***
5 mg kg^{-1} i.p. 15 min	4	[illegible]3.02 ± 0.87	3.86 ± 0.27	315 ± 40*	1[illegible].2 ± 1.1
5 mg kg^{-1} i.p. 30 min	10	23.02 ± 0.44	3.38 ± 0.30*	388 ± 37*	13.1 ± 0.5***
5 mg kg^{-1} i.p., 60 min	6	[illegible]3.3 ± 1.31	3.99 ± 0.46	482 ± 59	19.2 ± 1.[illegible]***
15 mg kg^{-1} i.p. 30 min	6	25.25 ± 1.55	2.87 ± 0.31	682 ± 61	19.6 ± 1.[illegible]***
Series B					
Control	9	[illegible]2.09 ± 0.27	8.42 ± 0.51	112 ± 13	8.9 ± 0.4
Amphetamine					
0.25 mg kg^{-1} i.v. 10 min	4	22.59 ± 0.27	4.06 ± 0.62*	285 ± 40*	10.9 ± 0.5**
2.5 mg kg^{-1} i.v. 10 min	4	[illegible]2.01 ± 0.58	4.05 ± 0.72	331 ± 78*	11.7 ± 1.[illegible]*
25 mg kg^{-1} i.v. 10 min	4	22.36 ± 0.78	2.89 ± 0.33	451 ± 53	12.4 ± 1.[illegible]**
2.5 mg kg^{-1} i.p., 10 min	4	20.99 ± 1.17	3.05 ± 0.55	345 ± 46	9.5 ± 0.8
5 mg kg^{-1} i.p., 30 min	4	22.65 ± 0.86	4.23 ± 0.92	274 ± 16	11.0 ± 1.3
5 mg kg^{-1} i.p., 60 min	5	22.71 ± 1.24	4.61 ± 0.90*	296 ± 79*	11.1 ± 1.0*

...ults confirm those obtained with the ^{133}xenon technique in showing that amphetamine ...se to a marked increase in CBF. However, in contrast to hypercapnia which in... CBF by similar degrees in all regions studied, amphetamine increased CBF much ... frontal (and parietal) cortex than in other regions. It should be emphasized that, ...ng 15 mg kg^{-1} of amphetamine, rCBF in frontal cortex was equal to that observed ... hypercapnia.

Discussion

...l discuss the present results under the headings (1) CBF and CMR_{O_2}, and (2) cerebral ...olic state.

...BF and CMR_{O_2}

...resent results have confirmed our preliminary observation that amphetamine sulphate ...es of 2.5–15 mg kg^{-1} gives rise to an increase of cortical CBF in paralyzed and artifi... ventilated rats. However, the increase in flow was less pronounced than previously ...ted and, accordingly, there was only a moderate rise in CMR_{O_2}. In fact, some animals ...ed no increase at all in CMR_{O_2}. The results raise the question whether or not methodo...al factors are involved, i.e. if the previous CBF method grossly overestimated CBF at ...igh flows encountered. As stated under Methods, the present modification of the ...non technique involves omission of any lag in sampling of blood, following start of ...uration, and more frequent sampling. This technique has now been applied to two ...r high-flow situations, i.e. hypoxia and hypercapnia (Berntman et al. 1978). The data ...ined allow an approximate recalculation of CBF values obtained, using the previous ...lag" procedure. The results show that the previously used technique can only have over...mated CBF by about 15%. Thus, differences in CBF and CMR_{O_2} between the present ...t, and the previous one, can hardly be attributed to methodological factor; hence the ...ts point to a difference in response to amphetamine. In support of our assumption, ...mals belonging to the new strain showed some difference in response to amphetamine ...hate, including an attenuated rise in blood pressure and less pronounced hyperventila...

...Measurements of regional CBF, using a completely different technique, corroborated ...flow data obtained with the ^{133}xenon method, and these expts. gave the interesting in...mation that CBF did not increase homogeneously, the most marked rise in CBF oc...ring in frontal, cortical regions. This is in contrast to hypercapnia (or hypoxia, see Salford ...d Siesjo 1974) which is associated with a more uniform increase of rCBF. The results ...o showed that, following 15 mg kg^{-1} of amphetamine, frontal rCBF rose to values ob...rved in animals maintained at an arterial P_{CO_2} of about 75 mmHg. Thus, the increase in ...BF induced by amphetamine is pronounced. It is of interest that amphetamine in doses of ...5 mg kg^{-1} i.e. has recently been shown to induce blood–brain barrier dysfunction and that ...rotein extravasation is confined to frontoparietal regions (Carlsson and Johansson 1977). Previous studies in man (Abreu et al. 1949, Shenkin 1951) ha... failed to indicate that amphetamine increases CBF (or CMR_{O_2}) but the doses employed were much smaller than

TABLE VI Regional cerebral blood flow (ml 100 g^{-1} min^{-1}) in different supra- and infratentorial [illegible] arterial blood pressure (mmHg) and arterial CO_2 tension (mmHg) are also given [illegible]

Groups	MABP	Pa_{CO_2}	Cortex			
			Frontal	Parietal	Temporal	[illegible]
Control	161 ±5	36.7 ±1.2	117 ±1	179 ±1	[illegible] ±1	[illegible]
Hypercapnia	166 ±5	72.1 ±2.8	435 ±95	496** ±92	321*** +41	[illegible]
Amphetamine						
2.5 mg kg^{-1} i.v. 5 min	158 ±8	38.9 ±1.2	348 ±55	320* ±78	142* ±21	[illegible]
5 mg kg^{-1} i.p. 30 min	166 ±7	40.1 ±1.1	284 ±51	303 ±47	142 +25	[illegible]
15 mg kg^{-1} i.p., 30 min	170 ±9	40.1 ±1.2	467* ±91	274 ±31	142* ±28	[illegible]

those used presently. Since our preliminary observations were published, there have appeared 3 other reports, indicating that amphetamine may increase glucose consumption in the rat brain (Neuser and Hoffmeister 1977), or CBF and CMR_{O_2} in the monkey brain (McCulloch and Harper 1977; McCulloch *et al.* 1977). CBF and CMR_{O_2} were increased by 50 and 15 per cent, respectively after *i.v.* injection, while, in the latter study the results suggest that increases in CBF and CMR_{O_2} are obtained only with small doses (0.75 nmol kg^{-1} min by intracarotid infusion) while larger doses (250 nmol kg^{-1} min^{-1}) have the opposite effect. In view of the differences in route administration, and in species, a direct comparison is difficult. However our results failed to indicate that such effects occur. Rather they show that effects on CBF and CMR_{O_2} are, if anything, directly proportional to the dose.

In the expts. of Nahorski and Rogers (1973), energy flux was calculated from rate of changes in the metabolites of the energy reserve, following decapitation of unanesthetized mice. In view of the fact that there may have been changes in body temperature (see Nahorski and Rogers 1975), the validity of the calculated energy flux values is not known. There are thus few data on cerebral metabolic rate in amphetamine-injected animals. The best interpretation of those available is that amphetamine induces a moderate increase in metabolic rate. Thus, CBF increases out of proportion to the rise in metabolic rate.

(2) *Cerebral metabolic state*

Published results on effects of amphetamine on cerebral metabolites are somewhat inconsistent. As remarked in the introduction, most workers have observed a decrease in glycogen concentration following amphetamine administration. The present results amply confirm these observations. In confirmation of some previous reports (Nahorski and Rogers 1975; King *et al.* 1975) our results also show that there were no changes in tissue concentrations of PCr or ATP. Our results offer the advantage that animals were studied after both short and long periods, following injection of amphetamine in [illegible] and that ADP

...are compared to one control group and one hypercapnic group. Six animals in all groups. ... ± S.E.

	Nucleus caudatus	Cerebellum	Pons	Medulla oblongata
	87 6	67 ±3	73 ±5	76 ±7
	216* ±39	229*** ±33	254** ±62	261 ±78
	166 ±40	107* ±22	102 ±21	120* ±20
	132* ±22	138* ±21	114* ±13	101 ±8
	129 +11	107* ±14	100 ±11	97 ±9

...MP concentrations were measured as well. Clearly amphetamine administration has ...ect on cerebral energy state.

...as previously been reported that amphetamine administration is followed by increases ...ue concentrations of lactate and malate (Nahorski and Rogers 1973, 1975). The in...e in malate, but not in lactate, was confirmed by King *et al.* (1975) who also noted an ...se in alanine concentration in mice, injected with amphetamine in a dose of 5 mg kg⁻¹ ...ew of the fact that amphetamine-injected animals may hyperventilate, and that hyper...lation by itself leads to increases in tissue concentrations of lactate, pyruvate, most ...acid cycle intermediates, and alanine (MacMillan and Siesjö 1973 Carlsson *et al.* 1975 ...berg 1976), such changes cannot without further proof be attributed to direct effects of ...hetamine in brain metabolism.

...e present expts. were conducted at constant P_{CO_2} (...nd body temperature), facilitating ...ation of amphetamine effects on metabolic pathways. Apart from the glycogenolysis, ...e were few effects of amphetamine in doses of 5 mg kg⁻ or less, when animals were ...led within 30 min following injection. The increases in G-6-P and α-KG concentrations ...not be interpreted with certainty. Thirty min following the administration of amphet...ine in a dose of 15 mg kg⁻ there were moderate increases in lactate concentration and ...ate/pyruvate ratio, and 60 min after a dose of 5 mg kg⁻¹ there was also an increase in the ...centrations of pyruvate, measured citric acid cycle intermediates, and alanine. Pre...mably the increase in pyruvate concentration reflects a certain mismatch between rates ...glycolysis and of pyruvate oxidation, while the increase in pyruvate by itself may explain ...y citric acid cycle intermediates and alanine accumulate. This is because anaplerotic ...rboxylation of pyruvate at the pyruvate carboxylase step depends on the pyruvate con...tration, and because an increased pyruvate concentration usually leads to a shift in the ...nine aminotransferase reaction towards alanine formation (see Norberg and Siesjö 1975).

Some of the effects observed after amphetamine administration, ... the decrease in gly...ogen concentration and the increases in the concentrations of pyruvate, lactate, citric acid ...cycle intermediates, and alanine, are similar to those occurring in seizures, ... those in-

duced by bicuculline. However in the latter condition the increased metabolic rate leads to perturbation of the phosphorylation state of the tissue, and to a large decrease in the tissue to blood glucose concentration ratio (*e.g.* Chapman *et al.* 1977). In amphetamine-injected animals there was no change in cerebral energy state, and the tissue to blood glucose concentration ratio did not fall. These facts probably reflect the moderate increase in metabolic rate following amphetamine administration.

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1 Changes in the respiratory rate, EEG activity, $[K^+]_e$ in the brain cortex, DC-potential and in blood pressure in control rat following cardiac arrest induced by i.v. injection of 0.3 ml of saturated $MgCl_2$ solution. The ensuing cerebral ischemia evoked transient increase in $[K^+]_e$ concomitant with an augmented amplitude in the EEG. Following this event the rate of rise in $[K^+]_e$ became steadily faster and after 108 s at $[K^+]_e$ of 8.6 mM there was rapid increase in $[K^+]_e$. At the same time the DC-potential showed a negative deflection. The EEG became flat after 18 s while the respiratory activity stopped after 10 s, returned after pause with series of gasps.

pretreatment. They were fasted overnight before the expts. They were anesthetized with pentobarbital (50 mg/kg). A cannula was inserted in the trachea and thin polyethylene catheters were placed in the femoral artery and vein for recording of the blood pressure and for infusion, respectively. The body temperature was maintained at 37°C with servo-controlled heating system. The animals were breathing spontaneously and the respiratory rate was recorded with thin thermosensor inserted in the tracheal tube. An ECG was recorded from needles in the two front legs. The head was immobilized in stereotaxic frame and trephine opening was made in the left parietal bone. The dura was carefully pierced with sharp needle and potassium-selective microelectrode was lowered into the cortex till final depth of approximately 0.4 mm by aid of motor driven micromanipulator. Double-barrelled potassium selective microelectrodes were made and used as described by Hansen (1977). The tip diameter was about 1 μm and the impedance of the barrel containing the potassium exchanger (Corning 477317) was 10^9–10^{10} Ohm. The response time (10%–90%) of the electrodes was 200–400 ms. The DC-potential was recorded between the reference barrel of the K-electrode and the common ground placed in neck muscle. The EEG was recorded differentially between 2 silver pins positioned on each side of the drilled hole and the common ground. The different parameters were recorded simultaneously on multi-channel U.V. light recorder (Southern Instruments).

Brain ischemia was produced by injection of 0.3 ml of saturated solution of $MgCl_2$ through the femoral vein. By this procedure the heart was arrested instantaneously without $MgCl_2$ reaching the capillary bed of the brain (Hansen 1977).

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The extracellular potassium concentration in brain cortex following ischemia in hypo- and hyperglycemic rats

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Abstract

HANSEN A J *The extracellular potassium concentration in brain cortex following in hypo- and hyperglycemic rats* Acta physiol scand 1978. *102* 324-329.

The extracell lar potassium concentration, $[K^+]_e$, was measured in the brain cortex of hypo- a hyperglycemic rats following brain ischemia. The increase in $[K^+]_e$ in control rats could be ch by 3 phases an initial slow rate of rise where the $[K^+]_e$ rose in min from 3 to 9 mM follo brupt, steep increase to 60 mM within 10 s and finally a slow rise to 80 mM. I the hyper glycemic rats the same pattern appeared, but there were significa t differences in the rate o d ration of the initial phase was pproximately doubled in the hyperglycemic and halved in the mic group The $[K^+]_e$ at which the steep increase was elicited was 8–10 mM i all groups. It that the duration of the initial phase is dependent upon available stores of glucose in the brain

It has been shown that the extracellular potassium concentration, $[K^+]_e$ in the bra exhibits a large increase following anoxia (Vyskočil *et al* 1972, Morris 1974 Hans The rise in the adult rat is characterized by an initial slow rate of rise followed by a steep increase, where the $[K^+]_e$ rises to 70 mM Finally a slow rise to about 90 mM The rate of rise was slower in the juvenile rat especially the duration of the i was longer and inversely related to the age (Hansen 1977) The longer duration was to lower metabolic demands of the juvenile brain and a greater tolerance of the anoxia. In this way the brain might maintain a sufficient ATP level for a longer thereby counteract the passive leak of potassium from the cells. However since bo ture and function of the nervous system rapidly changes during ontogenesis other isms might be important A study was therefore undertaken to clarify the impor the energy stores in the brain for the changes in $[K^+]_e$ during ischemia in the adu preliminary report has been given at the XXVIIth International Congress of Phys Sciences in Paris 1977

Methods

The experiments were performed on male Sprague Dawley rats, weighi g about 500 g. One gro was made hyperglycemic with an i.p. injection of 3 ml 50 glucose, 30–45 min before ische another group was made hypoglycemic with i.p. i jectio of 3 I U./kg of stalline porci (Leo) 2.5 h before ischemia. Untreated rats served as controls. All rats ically

1 $[K^+]_e$ in the rat cerebral cortex following ischemia. Effects of pretreatment with either glucose or insulin on the rise in $[K^+]_e$, EEG and respiratory activity following cardiac arrest (means ± S.E., number of animals in parentheses).

	Hypoglycemia (5)	Control (7)	Hyperglycemia (6)
glucose concentration (mM)	1.8 ± 0.22**	6.6 ± 0.83	29.0 ± 3.6
$[K^+]_e$ (mM)	3.0 ± 0.49	3.1 ± 0.18	3.2 ± 0.3
at the start of the steep increase (mM)	8.1 ± 0.78	9.4 ± 0.48	9.5 ± 0.4
... rate of rise in $[K^+]_e$ ($mM \cdot sec^{-1}$)	76 ± 11	74 ± 3.4	20 ± 2.8*
$[K^+]_e$ (mM)	75 ± 1.6	79 ± 2.0	74 ± 1.5
until start of the steep increase (sec)	39 ± 4.3***	105 ± 6.9	208 ± 11.6
... until flat EEG (sec)	14 ± 1.0**	19 ± 0.9	22 ± 0.6**
... until final gasp (sec)	52 ± 1.3*	82 ± 2.7	10[illegible] ± 7.1

...ces. different from control group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

...e terminal value of $[K^+]_e$. The most pronounced difference was found in the dura- ... of the initial, slow increase in $[K^+]_e$. In the hyperglycemic group the initial phase was ...e as long as in the controls while in the hypoglycemic group it was less than one half ... highest rate of rise during the steep increase, which in all groups was attained at a ...$]_e$ of 30-40 mM, was similar in the normo- and the hypoglycemic groups, but signif...tly lower in the hyperglycemic group. The duration of the EEG during ischemia and ...time to the last gasp were also influenced by the pretreatment.

Discussion

...s study has shown that altering the plasma glucose level alters the rate of rise of the ...tial, slow increase and the steep increase in $[K^+]_e$ without changing the $[K^+]_e$ at which ...e steep increase begins. In the following the two phases will be discussed separately

...ow increase

...e variation in the plasma level of glucose was induced as described by Ljunggren *et al* ...974). They showed in similarly pretreated rats that the brain glucose level increased 3-fold ...s rats given glucose and decreased to about 1/10 of the normal value in those receiving ...insulin. Since glucose is an important energy substrate during anaerobic conditions, re...eneration of ATP should be different in the three groups. Accordingly Ljunggren *et al.* ...1974) showed that the rate of lactate accumulation in the brain cortex following ischemia, ...eflecting the anaerobic production of ATP was larger in the hyperglycemic rats and lower ...n the hypoglycemic rats, than in controls. In another study Hansen and Nordström (in ...preparation) compared the $[K^+]_e$ in the brain cortex and the level of cortical energy stores ...phosphocreatine, ATP ADP glucose and glycogen) during ischemia in normal juvenile ...rats and juvenile rats with a high level of glucose in the brain. They found that the rate of ...rise in $[K^+]_e$ was lower and the cortical energy consumption, measured as the rate of decline ...in energy stores, concomitantly was higher in the group with high brain glucose level, than ...in controls, suggesting that the lower rate of rise in $[K^+]_e$ was due to a higher activity of ion

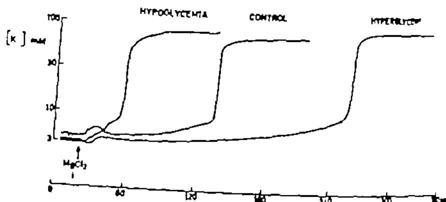


Fig. 2. Examples of recordings of [K]ₑ in the brain cortex of three rats following cardiac arrest by i.v. injection of a saturated $MgCl_2$ solution. In order to change the glucose stores in the brain an pretreated with either glucose or insulin. The rate of rise in [K]ₑ following cerebral ischemia was dependent upon the pretreatment. Note that the [K]ₑ at the beginning of the steep increase is the in all three animals.

After the electrode was inserted into the brain corte at least 5 min elapsed at hich the potassium centration was con tant. A few minutes before the induction of ischemia 0.5 ml of blood s sampled the femoral artery. The glucose concentration in the serum was determined by a glucose oxidase me The results were statistically evaluated using Student s t-test.

Results

Fig. 1 shows an expt. from a control rat where brain ischemia was induced without pr changes in the plasma glucose level. The resting level of [K]ₑ was 3.6 mM. Follow ischemia the [K]ₑ level initially exhibited a transient increase, presumably due to rele of potassium from the nerve cells as evidenced by the augmented amplitude in the EE After about 1 min there was a progressive rate of rise in [K]ₑ and after 2 min, at a [K] of 9 mM a rapid rise took place. During this event the [K]ₑ increased to 60 mM withi From then on the rate of rise became slower and a terminal value of 85 mM was reach The DC-potential showed a positive-going shift during the initial rise in [K]ₑ (expe during the transient increase in [K]ₑ). Concomitant with the rapid rise in [K]ₑ the DC potential showed a negative shift, so-called anoxic depolarization (Leão 1947). The EE exhibited an increased amplitude immediately following the injection of $MgCl_2$ and becam flat after 18 s. The respiration stopped after 10 s. After a pause it started again with a seri of gasps.

Fig 2 shows the change in [K]ₑ following ischemia in individual rats belonging to the three groups. It can be seen that the initial rise is clearly dependent upon the pretreatment. Thus, both the duration and the rate of rise was influenced by the pretreatment. Despite a significant difference in the duration of the initial phase the steep increase was elicited in al groups when the [K]ₑ had reached a value of 8–10 mM.

Table I summarizes the different parameters measured in the three groups prior to and during ischemia. The treatment with either glucose or insulin induced large changes in the plasma glucose level. On the other hand, it did neither affect the resting level of [K]ₑ nor the [K]ₑ at which the steep increase started during ischemia. Nor re any differences

here are several suggestions as to a possible mechanism behind the dramatic increase [illegible]]$_e$, but real insight into the chain of events is lacking.

technical assistance from Mrs Marianne A. Sørensen is gratefully acknowledged.

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pumping. Accordingly the inverse relation between the rate of rise in [K]$_e$ and the plasma glucose level observed in this study is probably caused by differences in ion pump activity

Steep increase

Since the length of the initial phase was related to the plasma glucose level, and thereby the glucose level in the brain, it is possible that the steep increase was elicited by a complete stop of ion pumping due to substrate failure. Data on this matter is, however lacking. It is tempting to try to assess the changes in [K]$_e$ caused by termination of ion pump activity

Suppose that 90 of the ATP hydrolysis in the brain cortex (30 μmol g min^{-1} ... *et al* 1973) is used for maintaining ion gradients. The Na -K pump uses 1 ATP molecule for transporting 3 sodium ions out of the cell and 2 potassium ions into the cell. The pump then counteracts a leak of potassium from the cells which in an extracellular space of ... would lead to an increase of [K]$_e$ with a rate of 6 mM s (30 0.9 2 ...). The calculated figure is clearly lower than the experimentally determined rate of rise (cf Table ...). It is obviously only valid for the assumed size of the extracellular space and for the membrane potential and the membrane permeability to potassium existing in the undisturbed cortex. Changes in these parameters would affect the rise in [K]$_e$. The extracellular space in the brain shrinks to 1/2–1/3 during anoxic depolarization (i.e. the steep increase) (Van Harreveld 1972), but this change is too small to account for the observed rate of rise of [K]$_e$. Changes in ionic permeability of the cell membrane is another possibility. An isolated increase in potassium permeability cannot explain the steep rise since potassium is distributed across the cell membranes near electrochemical equilibrium, and net-potassium efflux is therefore small until significant depolarization has taken place. A depolarization of the cells would require sodium influx. In accordance with this, during a spreading depression, where a similar steep increase in [K]$_e$ is observed (Vyskočil *et al* 1972) there is a considerable decrease in the extracellular sodium concentration (Kraig and Nicholson 1976). Thus an increase in the sodium permeability could provide a basis for the steep increase. This increase in sodium permeability could be mediated by the increase in [K]$_e$ depolarizing the nerve cells (Hodgkin and Huxley 1952). The observation that the [K]$_e$ was the same in all groups at the beginning of the steep increase supports this idea. The "threshold" value of 8–10 mM is in accordance with the findings of Vyskočil *et al* (1972) who produced brain anoxia by respiratory arrest and with Hansen (1977) who used nitrogen inhalation. The fact that when the [K]$_e$ in the brain cortex exceeds 10–12 mM during electrical stimulation a spreading depression is elicited (Lothmann *et al* 1975) also corroborates this view

The reason why the maximal rate of rise was considerably lower in the hyperglycemic rats than in the other groups is so far unknown. It might be speculated that the population of cells which delivers the potassium during the steep increase is inhomogenous. When the initial phase is prolonged the release of potassium occurs at different time leading to a lower rate of rise.

This discussion has focused upon potassium as the mediator of the steep increase, but the possibility remains, however that the metabolic breakdown damages the membrane integrity thus leading to unselective permeability changes striking glial cell as well as neurons.

... was exclusively carried by Ca ions. The spike frequency increased by application ... otropin releasing hormone (TRH), which increases prolactin secretion from these ... Dales *et al.* (1977) have also reported that cultured cells from anterior pituitary ... n, including the GH line, human acidophilic and chromophobic adenoma, generate ... potentials. However the action potentials of the GH cells were in this case shown to ... oth Ca- and Na-dependent components.

... n these studies on neoplastic tumour cells from the anterior pituitary gland two ... ons emerge: (1) Have normal anterior pituitary cells the ability to generate action ... tials? (2) If so, which ions act as inward current carriers?

... have examined the membrane electrical properties of anterior pituitary cells of the ... l rat. The present results show that most anterior pituitary cells are electrically ... ble, and that the action potentials are partly dependent on Ca ions. A brief report on ... of the present results has already appeared (Ozawa and Sand 1977).

Methods

... terior lobe of the pituitary gland was removed from female rats (Wistar strain) weighing 200–250 g. ... l slices of 300–500 μm thickness were prepared from each hemisphere of the anterior lobe. The ... que was essentially similar to that commonly used for making hippocampal slices. From this latter ... ration it ... possible to obtain *in vitro* intracellular recordings from the pyramidal cells showing action ... tials and synaptic potentials ... hich do not differ from those observed *in vivo* (Yamamoto 1972). Prior ... experiments the slices were incubated at 37°C for 2 h in ... buffer gassed with either ... mixture of ... O_2 and 5% CO_2 or pure oxygen. A selected slice was then transferred to the 0.4 ml superfusion ... r for ... here the slice was clamped on ... stainless steel mesh covering the outlet hole at the bottom. ... flow rate of the oxygen-saturated buffer through the chamber ... as 1 ml/min, and the position of the ... ation facilitated solution exchange ... than the slice. The temperature of the perfusing solution ... as ... tained at 37 ± 1°C.

... e composition of the normal buffer was NaCl 124 mM, KCl 5 mM, NaH_2PO_4 1.24 mM, $NaHCO_3$ ... mM, $MgCl_2$ 1.3 mM, $CaCl_2$ 2.4 mM and glucose 10 mM. This solution was gassed with 95 ... O_2 and ... CO_2. Most experiments were, however, performed in standard buffer of the following composition: ... l 130 mM, KCl 5 mM, $MgCl_2$ 1.3 mM, $CaCl_2$ 2.4 mM, glucose 10 mM and buffered by Tris-HCl 5 mM ... pH 7.4. This solution was gassed with pure oxygen. No difference was observed between the results ... ined in these two solutions. Na-free solution ... as made by replacement of NaCl of the standard ... tion ... th Choline-Cl. Solution ... ith high Ca^{++} concentration was prepared by substitution of either ... Cl or Choline-Cl for $CaCl_2$ on an isotonic basis. Ba and Sr solutions were made by replacement of ... Cl_2 with $BaCl_2$ and $SrCl_2$, respectively.

... Glass micro-electrodes for recording cellular transmembrane potentials were filled from behind by ... illary action with 4 M K-acetate solution adjusted to pH 7.2 with acetic acid. The electrode resistance ... ged between 70 and 120 MΩ. The electrodes ... ere connected to ... high input impedance preamplifier ... h the facility for injecting current through the recording electrode using ... bridge circuit (Biodyne ... ctronics Laboratory ... AM 1). The input resistance of the preamplifier was more than 10^{11} MΩ and ... input offset current ... as less than 10^{-11} A. The time derivative of the potential change was recorded ... an electronic differentiation circuit.

Results

... Electrotonic responses in standard solution

... ward current pulses of 200–300 ms duration and less than 1 ... 10^{-10} A intensity were ... continuously injected during advancement of the microelectrode through the anterior ... pituitary slice. Successful penetration of a cell was noticed by sudden appearance of the

Acta physiol scand 1978 102, 330-341

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Electrical activity of rat anterior pituitary cells *in vitro*

By

SEIJI OZAWA and OLAV SAND

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Abstract

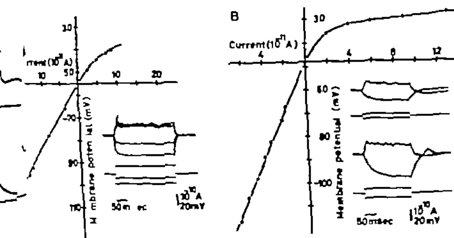
OZAWA, S and O SAND *Electrical activity of rat anterior pituitary cells in vitro* Acta physiol scand. 1978 *102* 330–341

Regenerati e responses were seen in most cells either after cessation of an inw rd current pulse or during an outward current pulse Two cell groups were distinguished electrophysiologically Type I cells showed action potentials with maximum rate of rise f 21.2 ± 9.0 V/sec (mean ± S.D n = 19), [illegible] type II cells generated small graded depolarising responses with maximum rate of rise less than 3 V/s The [illegible] potentials of type I and II cells were 31.8 ± 14.9 mV (n = 19) and 41 7 ± 9.8 mV (31), respectively. The steady-state current/voltage relationship wa linear for both cell types when the membrane potential was more negative than − 60 mV An outw rd rectification appeared when the membrane potential was more positive than − 40 mV The input resistance was smaller in type I cells (274 ± 212 MΩ, [illegible]) than type II cells (1 11 ± 456 MΩ, n = 16). E en in Na free solution regenerati responses [illegible] observed in most cells. When the Ca^{++} concentration was increased tenfold to 4 mM, the maximum rate of rise of the off-response increased from 1 9 ± 0.8 V/s (n = 11) to 5 7 ± 2.12 V/s (= 5). All-or-none action potentials could be evoked in this Ca^{++} rich solution Action potentials of similar maximum rate of rise could be evoked after replacing 4 mM Ca with isomolar Sr^{++} Prolonged action potentials were seen after substitution of Ca^{++} for Ba It is concluded that action potentials in most anterior pituitary cells have a Ca component, which in type I cells is additional t a Na component.

Key words Anterior pituitary action potential, Ca spike hormone secretion

It has recently been demonstrated that endocrine cells from the pancreatic islets (β-cells) and the adrenal medulla (chromaffin cells) are capable of generating action potentials (Dean and Matthews 1968 1970 a, Biales *et al* 1976) These action potentials are due at least partly to an increase of the membrane permeability to Ca ions (Dean and Matthews 1970 b, Brandt *et al* 1976) Presence of Ca ions in the extracellular solution is indispensable for hormone secretion, and it is generally accepted that entry of external Ca ions into the cells triggers exocytosis of the secretory granules. It is therefore reasonable to suggest that the Ca spike generating mechanism of these cell membranes has important functional significance in the stimulus-secretion coupling.

Regarding the anterior pituitary cells, Kidokoro (1975) observed spontaneously occurring spikes in a clonal cell line (GH_3) isolated from a rat anterior pituitary tumour which continuously secretes growth hormone and prolactin. He concluded that the inward action



Steady-state current/voltage relations for type I (A) and type II (B) cells in standard saline. The ...ane potential changes measured at the end of the current pulses were plotted. Inward current is ...t on the abscissa. The resting potential was −55 mV and −48 mV in A and B, respectively. The ...resistance estimated from the linear portion of the I/V relation was 275 MΩ in A and 1 080 MΩ ...Inset. Sample records showing time course of the potential change. The potential difference across ...recording electrode was not completely cancelled in record A, and corrected values were therefore ...d on the graph

...ward current pulses in type II cells, but small graded responses with the appearance of ...ped oscillations were instead superimposed on the electrotonic potential. The resting ...entials of type II cells were more negative (41.7 ± 9.8 mV, n = 31) and generally more ...le than for type I cells. The penetrations frequently lasted for several minutes.

...mbrane resistance and capacitance

...steady state relation between the current intensity and the resulting voltage deviation ...the end of injected current pulses of 200–300 msec duration was obtained for both type ...and II cells in standard saline (Fig. 2A & B). The relation was linear for both groups ...en the membrane potential was more negative than −60 mV and the slope resistance ...nded to decrease when the membrane potential was made more positive than −40 mV ...terior pituitary cells thus have the property of outward rectification seen in most ...citable cells. This outward rectification was more pronounced in type II cells. The input ...sistance was estimated from the linear portion of the current/voltage relation. Although ...e value for type I cells scattered widely from 92 MΩ to 960 MΩ (274 ± 212 MΩ, n = 19), ...is in the same range as the values reported by Kidokoro (1975) and Biales *et al.* (1977) for ...H clonal cell lines. There was clear correlation between the resting potential and the ...put resistance in type I cells. The input resistance decreased with less negative resting ...otentials, probably due to increase in leak conductance following cell damage caused by ...he penetration. Hence, the mean value of the input resistance of type I cells described ...above is possibly an underestimation of the true value. The resting potentials of type II ...cells were less scattered and there was no correlation between the resting potential and the

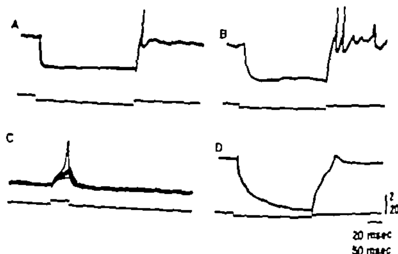
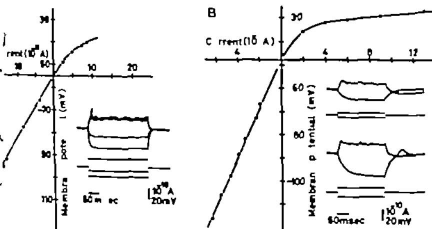


Fig. 1 Intracellular records of regenerative responses in standard saline. A and B, responses (type I) elicited at the termination of an anodal current pulse C, action pote short outward current pulse. D slow regenerative response (type II). The time sc applicable only to record C

negative DC membrane potential and the hyperpolarising electrotonic poten the inward current pulse In about 80% of the penetrated cells where these fulfilled regenerative responses were evoked either at the termination of the i pulse or during an outward current pulse (Fig. 1) However the regenera in the type II cells described below were rather fragile, and frequently fade the electrode was still inside the cell It is therefore doubtful if the remainin penetrated cells were really non regenerative. The present paper deals only w majority of cells which displayed regenerative responses.

The cells could be divided into two classes according to the characte regenerative responses. In one group of cells a regenerative spike with an amp to 45 mV (mean ± S D – 29 1 ± 10 3 mV n – 19) was evoked at the termin current pulse (Fig. 1A) The maximum rate of rise of the response ranged fro 40 V/s (21.2 ± 9 0 V/s, n – 19) Repetetive firing was seen in some cases (Fi responses were followed by afterhyperpolarisation. The resting potential o were usually in the range of – 20 to – 50 mV (31.8 ± 14 9 mV n – 19). The l the resting potential was possibly due to cell injury caused by the electrode . In agreement with this interpretation, the recordings from these cells were rath and usually lasted less than 1 min However three cells had resting potenti – 55 and – 65 mV and in these cells all-or none action potentials could be g outward current pulses in a similar way as usually seen in other excitable cells The cells capable of generating fast rising action potentials with maximum r more than 11 V/s will tentatively be referred to as type I cells in the present pa

Much smaller and more slowly rising graded regenerative responses were evo termination of the current pulse in the remaining responsive cells (Fig. 1 D). tentatively be called type II cells. The amplitude of these responses ranged betwee mV and the maximum rate of rise was less than 3 V/s Action pot not



...Steady-state current/voltage relations for type I (A) and type II (B) cells in standard saline. The ...ane potential changes measured at the end of the current pulses were plotted. Inward current is ...n on the abscissa. The resting potential was −55 mV and −48 mV in A and B, respectively. The ...sistance estimated from the linear portion of the I/V relation was 275 MΩ in A and 1 080 MΩ ...nset: Sample records showing time course of the potential change. The potential difference across ...ding electrode was not completely cancelled in record A, and corrected values were therefore ...l on the graph.

...rd current pulses in type II cells, but small graded responses with the appearance of ...ed oscillations were instead superimposed on the electrotonic potential. The resting ...tials of type II cells were more negative (41 7±9.8 mV n=31) and generally more ... than for type I cells. The penetrations frequently lasted for several minutes.

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...eady state relation between the current intensity and the resulting voltage deviation ...e end of injected current pulses of 200–300 msec duration was obtained for both type ...I II cells in standard saline (Fig. 2A & B) The relation was linear for both groups ...the membrane potential was more negative than −60 mV and the slope resistance ...ed to decrease when the membrane potential was made more positive than −40 mV ...rior pituitary cells thus have the property of outward rectification seen in most ...able cells. This outward rectification was more pronounced in type II cells. The input ...tance was estimated from the linear portion of the current/voltage relation. Although ...alues for type I cells scattered widely from 92 MΩ to 960 MΩ (274±212 MΩ, n=19), ...in the same range as the values reported by Kidokoro (1975) and Biales *et al* (1977) for ...clonal cell lines. There was a clear correlation between the resting potential and the ...t resistance in type I cells. The input resistance decreased with less negative resting ...ntials, probably due to increase in leak conductance following cell damage caused by ...penetration. Hence, the mean value of the input resistance of type I cells described ...e is possibly an underestimation of the true value. The resting potentials of type II ...s were less scattered and there was no correlation between the resting potential and the

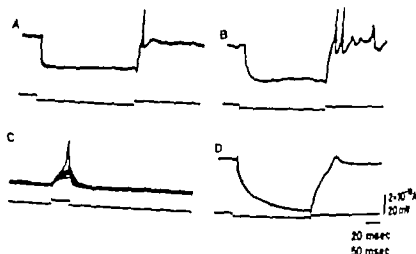
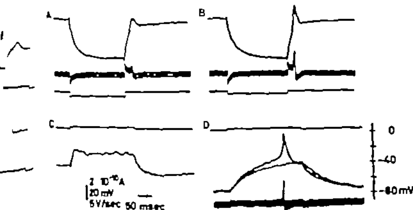


Fig. 1 Intracellular records of regenerative responses in standard saline. A and B, fast regenerative responses (type I) elicited at the termination of an anodal current pulse. C, action potential evoked by a short outward current pulse. D slow regenerative response (type II). The time scale of 20 msec is applicable only to record C.

negative DC membrane potential and the hyperpolarising electrotonic potential caused by the inward current pulse In about 80% of the penetrated cells where these criteria were fulfilled regenerative responses were evoked either at the termination of the inward current pulse or during an outward current pulse (Fig. 1). However the regenerative responses in the type II cells described below were rather fragile, and frequently faded away while the electrode was still inside the cell It is therefore doubtful if the remaining 20% of penetrated cells were really non regenerative. The present paper deals only with the majority of cells which displayed regenerative responses.

The cells could be divided into two classes according to the characteristics of the regenerative responses. In one group of cells a regenerative spike with an amplitude up to 45 mV (mean ± S D = 29 1 ± 10.3 mV n = 19) was evoked at the termination of the current pulse (Fig. 1A) The maximum rate of rise of the response ranged from 11 to 40 V/s (21 ? ± 9 0 V/s n = 19) Repetetive firing was seen in some cases (Fig. 1B) responses were followed by afterhyperpolarisation. The resting potential of these cells were usually in the range of −20 to −50 mV (31 8 ± 14.9 mV n = 19). The low value of the resting potential was possibly due to cell injury caused by the electrode penetration. In agreement with this interpretation, the recordings from these cells were rather unstable and usually lasted less than 1 min. However three cells had resting potentials between −55 and −65 mV and in these cells all-or none action potentials could be generated by outward current pulses in a similar way as usually seen in other excitable cells (Fig. 1C). The cells capable of generating fast rising action potentials with maximum rate of rise more than 11 V/s will tentatively be referred to as type I cells in the present paper

Much smaller and more slowly rising graded regenerative responses were evoked at the termination of the current pulse in the remaining responsive cells (Fig. 1 D), which will tentatively be called type II cells. The amplitude of these responses ranged between 3 and [illegible] mV and the maximum rate of rise was less than 3 V/s Action [illegible] not evoked



Regenerative responses in Na-free solution. A, response to anodal current break in 2.4 mM Ca^{++}. B, corresponding response recorded from the same slice 45 min after introduction of 24 mM Ca^{++} ... C, membrane potential change in response to ... cathodal current pulse in 24 mM Ca^{++} solution ... after penetration. D, an all-or-none action potential was elicited by ... cathodal current pulse of ... intensity ... hen the membrane was hyperpolarised by 33 mV. The middle trace in A and B and the trace in D gives the first order derivative of the potential change.

... entration was in these expts. performed to wash out Na ions completely. Severa... cellular recordings from a selected slice were then obtained in this solution, where... the slice was superfused with Na-free solution containing 24 mM Ca^{++} (10 ... Ca... tion) for at least 30 min before the recordings were repeated. Both the amplitude and ... maximum rate of rise of the regenerative response increased remarkably in the Na-free ... tion of high Ca^{++} concentration, as illustrated in Fig. 4 A and B. The largest value ... he maximum rate of rise of the graded regenerative response which occurred at the ... ination of the inward current pulse was 1.9 ± 0.8 V/s (n = 11) in normal Ca^{++} concen... ion and 5.7 ± 2.1 V/s (... 5) in 10 ... Ca^{++} solution. Furthermore, all-or-none action ... entials could be evoked by depolarising pulses in the Na-free solution of high Ca ... centration ... hen the membrane potential was hyperpolarised to a level more negative ... n ... 75 mV by background current. The cell illustrated in Fig. 4 had a resting potential ... 44 mV shortly after the penetration and showed small graded depolarisations in ... ponse to an outward current pulse (Fig. 4 C). When the membrane potential was shifted to ... 77 mV by continuous inward current, an all-or-none action potential was generated by ... outward current pulse (Fig. 4 D). The action potential was unaffected by application of ... μM tetrodotoxin.

...ction potentials in Sr and Ba solution

...n most excitable tissues exhibiting a Ca-dependent regenerative response, Sr^{++} and Ba^{++} ...n substitute for Ca... as current carrier (Fatt and Ginsborg 1958). We therefore con... ucted experiments in which the 24 mM Ca... in Na-free solution was replaced with ...osmolar Sr^{++} or Ba^{++}. Regenerative responses similar to those observed in ...4 mM Ca^{++} ...olution ... ere recorded at the termination of the anodal current pulse in the Na-free

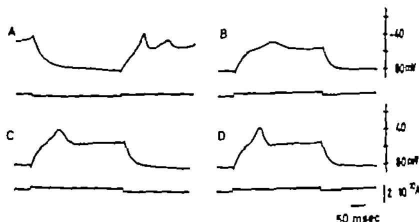


Fig. 3 Depolarising responses in Na-free solution containing 2.4 mM Ca^{2+} A, regenerative response recorded shortly after penetration. B, C and D records obtained while the membrane potential was held at −78 mV by DC hyperpolarising current The magnitude of the graded responses increased with increasing intensity of the pplied cathodal current pulses.

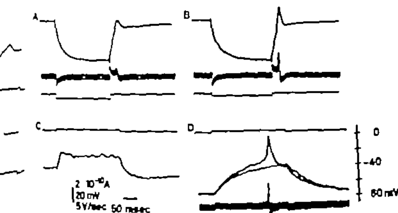
input resistance. These results probably reflect that type II cells were less damaged by the penetration The input resistance of type II cells averaged 1 112 ± 456 MΩ (n 16), which is four times larger than the underestimated value for type I cells.

The membrane time constants for the two cell types were estimated from the exponential time course of the potential change produced by a small inward current pulse (see sample records in Fig. 2) The values were 4 6 ± 3 0 ms (n ~ 19) and 29 1 ± 12.6 ms (n 16) for type I and II cells, respectively The corresponding membrane capacities obtained from the time course and the membrane resistance were 1 7 ± 1 0 10⁻ F (n 19) and 2.6 ± 1 6 10 F (n ~ 16)

Regenerative responses in Na-free solution

After superfusion of the thin slice with Na free solution containing 2.4 mM Ca^{++} for several minutes, fast rising regenerative responses similar to those of type I cells in normal buffer were never observed However the cells were still excitable in Na free solution. Successful penetrations of numerous cells with resting potentials of about −40 mV were performed, and a small but significant regenerative response almost always occurred at the termination of the inward current pulse (Fig. 3 A). In the cell illustrated in Fig. 3 the membrane potential was shifted from ~44 mV to −78 mV by a continuously injected DC inward current Outward current pulses of increasing intensity were then applied, and Fig 3 B-D show how these pulses produced significant depolarising responses of increasing magnitude superimposed on the passive electrotonic potential. Action potentials of all-or-none type could not be evoked in Na-free solution containing 2.4 mM Ca but the presence of a graded response suggests that a potential dependent increase of the membrane permeability to Ca^{++} may occur in anterior pituitary cells.

In order to test this possibility the effects of increased Ca concentration was studied. Pre incubation of the sliced anterior pituitary for 3 h in Na-fr ~ of 2.4 mM Ca^{++}



Regenerative responses in Na free solution. A, response to anodal current break in 2.4 mM Ca^{++}. B, corresponding response recorded from the same slice 45 min after introduction of 24 mM Ca^{++}. C, membrane potential change in response to cathodal current pulse in 24 mM Ca^{++} solution after penetration. D, an all-or-none action potential was elicited by a cathodal current pulse of intensity when the membrane was hyperpolarized by 33 mV. The middle trace in A and B and the trace in D gives the first order derivative of the potential change.

...stration was in these expts. performed to wash out Na ions completely. Several cellular recordings from a selected slice were then obtained in this solution, where the slice was superfused with Na-free solution containing 24 mM Ca^{++} (10 Ca^{++} ion) for at least 30 min before the recordings were repeated. Both the amplitude and maximum rate of rise of the regenerative response increased remarkably in the Na-free solution of high Ca^{++} concentration, as illustrated in Fig. 4 A and B. The largest value of the maximum rate of rise of the graded regenerative response which occurred at the termination of the inward current pulse was 1.9 ± 0.8 V/s (n = 11) in normal Ca concentration and 5.7 ± 2.1 V/s (n = 5) in 10 Ca^{++} solution. Furthermore, all-or-none action potentials could be evoked by depolarizing pulses in the Na-free solution of high Ca^{++} concentration when the membrane potential was hyperpolarized to a level more negative than 75 mV by background current. The cell illustrated in Fig. 4 had a resting potential of 44 mV shortly after the penetration and showed small graded depolarizations in response to an outward current pulse (Fig. 4 C). When the membrane potential was shifted to 77 mV by continuous inward current, an all-or-none action potential was generated by an outward current pulse (Fig. 4 D). The action potential was unaffected by application of μM tetrodotoxin.

Action potentials in Sr and Ba solution

In most excitable tissues exhibiting a Ca-dependent regenerative response, Sr^{++} and Ba^{++} can substitute for Ca^{++} as current carrier (Fatt and Ginsborg 1958). We therefore conducted experiments in which the 24 mM Ca in Na-free solution was replaced with equimolar Sr^{++} or Ba^{++}. Regenerative responses similar to those observed in 24 mM Ca^{++} solution were recorded at the termination of the anodal current pulse in the Na-free

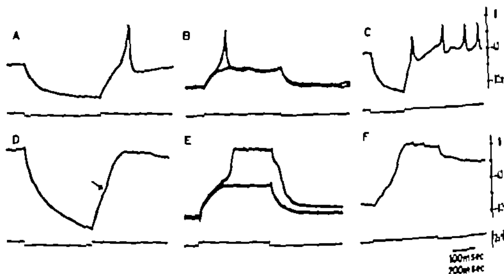
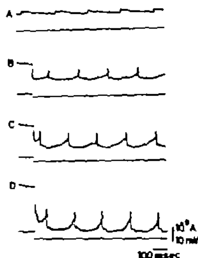


Fig. 5 A, B and C, action potentials in Na free, 24 mM Sr^{2+} solution. A, action potentials evoked at the termination of an anodal current pulse B, all-or-none action potentials elicited by a cathodal current pulse after the membrane potential was shifted to −75 mV by DC hyperpolarising current C, repetitive firing of a different cell after cessation of an anodal current pulse. The time scale of 200 msec is applicable only to this record D E and F regenerative responses in Na-free, 24 mM Ba^{2+} solution. D, anodal break response. Arrow indicates the inflection reflecting the critical membrane potential for the regenerative response. Note that the resting potential was positive in this cell. E, prolonged action potentials elicited by an outward current pulse after the membrane potential was shifted to −75 mV F prolonged regenerative response lasting about 1 s.

solution containing 24 mM Sr^{2+} (Fig. 5 A) The largest value of the maximum rate of rise of the regenerative response was 5 9 ± 2.5 V/s (n = 9) Repetitive firing occasionally occurred after the anodal pulse, as shown in Fig. 5 C Moreover an all-or-none action potential could be generated by an outward current pulse when the membrane potential was hyperpolarised to the level of −75 mV (Fig. 5 B)

In Na free solution containing 24 mM Ba the resting potential ranged from −25 to +20 mV and five out of twelve cells displayed positive values. A positive resting potential in 10 mM Ba^{2+} solution has previously been found in the rat chromaffin cell by Brandt *et al* (1976) Fig 5 D shows the response to anodal current break in Ba solution, and an inflexion is seen around the level of −40 mV during the repolarising phase. The inflexion may indicate the onset of a regenerative process dependent on Ba or in other words that the Ba permeability of the membrane increases when this potential level is reached. Prolonged action potentials could be evoked by outward current pulses in Ba-solution provided that the membrane potential was shifted to more negative values than −65 mV (Fig. 5 E and F) The threshold level of these action potentials coincided with the potential level where the inflexion of the repolarising phase following anodal current break was seen. This fact confirms the interpretation given above for this inflexion. The prolonged action potential often lasted more than one second (Fig. 5 F) This prolongation is probably due to a suppressive effect of Ba ions on the development of the delayed rectification, as reported for other cells (Werman and Grundfest 1961 Hagiwara and Naka 1964, Hagiwara and Kidokoro 1971)



A, spontaneous potential fluctuations in Na-free. Two potential components exist. B, C and D, the ...de of both components increased with increasing ...y of the applied anodal current pulses. Further de... in the text.

...taneous potential fluctuations

...ing advancement of the electrode through the pituitary slice we occasionally en...tered spontaneous depolarising potentials which occurred regularly at the frequency ...4 Hz, as illustrated in Fig. 6 A. These potentials could also be seen in Na-free solution. ...er inspection revealed that the potentials consist of two components. The first is a ...depolarisation on which the other faster rising and decaying potential is superimposed. ...e amplitude of both components increased when anodal current pulses of increasing ...nsity were applied through the recording electrode (Fig. 6 B–D). The records in ...6 clearly show that the spike potential is preceded by a slow depolarising potential, and ...at both components were monophasic depolarisations. In spite of this latter fact we ...sume these recordings to originate from incomplete cell penetrations for the following ...sons: (1) The resting potential was always less negative than −10 mV. (2) The ...parent input resistance measured by shift of the bridge balance ranged from 10 to 30 ...Ω, which is less than one-tenth of the value described above. (3) The potentials could ...recorded using an electrode with dull tip (resistance less than 40 MΩ), which made the ...viously described intracellular recordings impossible.

The nature of these potential fluctuations is essentially similar to that reported on ...ooth muscle cells of the rabbit colon (Gillespie 1962) and on cat lateral geniculate ...urons (McIlwain and Creutzfeldt 1967). The recordings in the latter studies were ...plained by a "quasi-intracellular" condition, where the electrode was sealed into an ...jured area of the membrane. The observed potential fluctuations will then reflect ...hanges in the intensity of the current passing across the sealing resistance, and the ...ecordings may show the true membrane potential change with some attenuation. Spon...aneous potential fluctuations could also be seen in ordinary intracellular recordings from ...cells in Na-free solutions containing high Ca^{++} or Sr^{++} concentration, which seemed to ...facilitate stable penetrations. Typical intracellular records from several cells superfused with

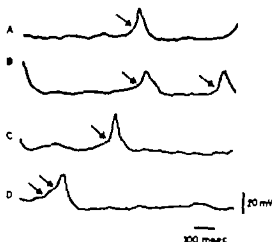


Fig. 7 Intracellular records of spontaneous ... potentials in Na-free, 24 mM Sr^{2+} solution. Succes... sive traces represent a continuous recording ... potentials were initiated by small depolari... fluctuations of the membrane potential ... indicate the transition between ... depolarisations and the action potentials ... two small depolarisations (the first arrow in D) ... resting potential was -35 mV and the spike generating mechanism was partially inactivated

these solutions displayed spontaneous depolarising potentials of less than 5 mV amplitud as shown in Fig. 7 Action potentials were occasionally initiated by these small depolarisa tions.

Discussion

Electrical excitability

In order to see the effect of hypothalamic extracts and other secretagogues for anterior pituitary hormones on the membrane potentials of the adenohypophyseal cells, several electrophysiological studies have previously been performed on the adenohypophysis of normal rats (Milligan and Kraicer 1970, Martin *et al* 1973 York 1974). Action potenti were however never observed in these studies. On the other hand Biales *et al.* (1977) h indicated that action potentials might occur in normal anterior pituitary cells, but evidence was presented

The present results clearly demonstrate that anterior pituitary cells in the normal are electrically excitable. The cells were electrophysiologically classified into two groups I cells have action potentials with maximum rate of rise up to 40 V/s, while only gr regenerative responses were observed in type II cells. Type II cells had more negative stable resting potentials as well as higher input resistance than type I cells. It is there reasonable to assume that type II cells were less injured by the electrode penetration type I cells, and we therefore reject the idea that the slowly rising regenerative respo are deteriorated action potentials of type I cells caused by poor penetrations. The corded cells were not identified histologically in the present study but the parench glandular cells overwhelmingly outnumber the other cell types within the gland. Reg ative responses were seen in most of the penetrated cells, and we therefore conclude the majority of the parenchymal glandular cells of the anterior pituitary are electr excitable.

The fast rising action potentials of type I cells were never obse ... a-free sol

ct indicates that these action potentials are mainly due to an increase of the membrane ability to Na ions. However significant regenerative responses were almost always Na-free solution at the termination of the inward current pulse. In Na-free solution ning 10 normal Ca^{++} concentration both the amplitude and the rate of rise of response increased, and all-or-none action potentials could be generated by outward t pulses. Action potentials could also be induced if the Ca of this solution was ed by isomolar Sr^{++} or Ba^{++} and the action potentials in all these solutions were nt to 6 μM tetrodotoxin. From these results we conclude that the membrane of anterior pituitary cells are capable of generating Ca dependent regenerative responses mura 1973), and that type I cells have a Na component in addition.

e electrical properties of the normal anterior pituitary cells demonstrated in the nt experiments are similar to those previously reported for clonal GH cells isolated a rat anterior pituitary tumour (Kidokoro 1975 Biales *et al* 1977) The present ts thus exclude the possibility that the ability of GH cells to generate action potentials, nd partly dependent on Ca might be caused by pathological changes of the neo-ic cells.

tional significance of the regenerative potentials

regenerative responses described in the present paper usually required current injec s to be evoked. It could therefore be argued that these responses might not occur under siological conditions. However our quasi-intracellular recordings revealed spon-ous depolarizing potentials. Corresponding observations were reported on GH_3 clonal lines by Kidokoro (1975) and on adrenal chromaffin cells by Brandt *et al* (1976). hese latter cases spontaneous action potentials were rarely seen in intracellular recordings, In spontaneous extracellular spikes were recorded from nearly every cell tested using a ro-suction electrode. It is therefore reasonably assumed that spontaneous regenerative entials occur in rat anterior pituitary cells under physiological conditions. In high or Sr^{++} solution spontaneous depolarising potentials were occasionally seen even using racellular electrodes

It has been suggested that the Ca-dependent regenerative responses in endocrine cells are ectly coupled to the hormone release. In other words, the entry of Ca ions into the lls during an action potential may trigger the hormone release under physiological condi-ons (Meissner and Schmelz 1974, Kidokoro 1975 Brandt *et al* 1976 Davis and Hadley 976). The hormone release from all the different types of secreting anterior pituitary ells requires Ca^{++} in the external medium (Kraicer 1975). The present demonstration that Ca-dependent regenerative responses could be evoked in nearly all of the penetrated cells consistent ith the view that such responses play an important role in controlling the hor mone secretion. Within the normal anterior pituitary *in vitro* only the prolactin and growth hormone secreting cells will release a significant amount of hormone in the absence of hypothal-amic factors (Vale and Rivier 1975). This is in agreement with the fact that only a small number of the penetrated cells displayed spontaneous depolarising potentials, although an additional explanation of this phenomenon has been given above. Future expts. should be performed to study the effects of hypothalamic releasing and release inhibiting factors on the

Ca-dependent regenerative responses of identified anterior pituitary cells, in order to study the functional significance of these responses.

Pancreatic islet β-cells, adrenal chromaffin cells and adenohypophysial cells have possibly a neural crest origin (Pearse 1970). Tischler *et al.* (1976) have demonstrated that all of the cultured human endocrine cells of proposed neural crest origin so far investigated are capable of generating all-or none action potentials. The question then arises if only endocrine cells of neural crest origin are excitable, or in other words if electrical excitability is linked to the embryological origin of the cell rather than to its function. The present results demonstrate that even anterior pituitary cells which have not developed from the neural crest are able to generate Ca-dependent regenerative potentials.

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Acta physiol. scand. 1978. 102. 342–356

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Blood flow distribution in the renal portal system of the intact hen. A study of a venous system using microspheres

By

Bo Odlind

Received 8 September 1977

Abstract

Odlind B *Blood flow distribution in the renal portal system of the intact hen. A study of a venous system using microspheres* Acta physiol. scand. 1978 102 342–356.

The blood flow in the renal portal system of intact hens was characterized by determining the fractional distribution of 15 µm and 50 µm microspheres in this system after injection into a leg vein. Validation showed that only 50 µm spheres gave a reliable estimation of this distribution. The blood flow in the coccygeomesenteric vein was directed towards the liver in nearly all cases. On the average 44, 47 and 1% of portal blood from the external iliac vein perfused the ipsilateral kidney the liver and the lungs, respectively. However the distribution of portal blood to these organs varied considerably between individuals and changed appreciably within 35 min in half the animals studied. The reason for these variations is not clear. The portal blood from the right and left external iliac veins was asymmetrically distributed in most hens, illustrating the importance of local factors in its regulation. Variation was great in the regional distribution of portal blood within the kidney possibly due to local vasoconstriction of portal vessels. Further information on the regulation of renal portal blood flow is needed to explain its physiological significance. Microspheres provide a convenient method for such studies. A combination of microsphere and Sperber techniques allows us to determine the renal excretion efficiency of a given substance.

Key words: Portal system, microspheres, birds, kidney phlebography venous circulation

The avian kidney is supplied by blood from the arterial and the renal portal circulation (Sperber 1948 a, Fig. 1) The physiological significance of blood flow in different parts of the renal portal system and its regulation is not fully understood (Gilbert 1961 Siller 1971). The dual blood supply to the avian kidney has been used extensively to study renal handling and effects of drugs, hormones and auto-pharmacological agents (May and Carter 1970, Wei... 1973 Rennick 1976) utilizing a technique developed by Sperber (Sperber 1946, 1948 a, ... There is, however an unpredictable variation in individual results obtained with the Sperber technique (Sperber 1948 b) which might be related to variations in blood flow in the re...

This work was presented in part at the XV Scandinavian Congress of Physiology and Pharmacology ... Aug. 1976, and published in abstract form (Acta physiol. ... Suppl. 440, ...

system (Akester 1964). The aim of the present investigation was to get quantitative ation about the distribution of blood flow in the renal portal system, under physiolo- onditions and in connection with the Sperber technique. Since the microsphere tech as chosen for this purpose, it was also necessary to test the validity of the technique applied to a venous circulatory system.

Materials and Methods

3 White Leghorn hens, 6–18 months of age and weighing between 1 1 and 1 4 kg, from 5 different ed dealers and one lot of non-laying White Leghorn pullets, 4–5 months of age and eighing between nd 1 1 kg were used The animals were kept on commercial feed for laying hens (Pulfor® Lant en, Sweden) but were fasted over night before the expts They had free access to tap water

osphere procedures

microspheres of 15 ± 5 μm diameter (mean ± S.D 3 M Co St. Paul, Minnesota, U.S.A), labelled 85-Sr and 3 batches of Tracer Sephadex microspheres (Pharmacia Fine Chemicals AB, Sweden) of 15 μm diameter (mean ± S D) were used. The large spheres (50 μm) were labelled with 57-Co or by procedure recommended by the manufacturer and ere carefully washed ith distilled ter and for more than week. The small spheres (15 μm) were subjected to homogenization shortly before injection in order to break up possible aggregates. The spheres were suspended in less than 1 ml of solution of Ficoll 70 (Pharmacia Fine Chemicals AB, Sweden) or in chicken plasma and were agitated iously prior to injection. The suspensions were injected at rate of 0 2–0 4 ml/min, followed by slow tion of 0 5 ml saline Animals were sacrificed 5 (3–5), 25 or 60 min after the injection, using 75–100 mg/ of sodium pentobarbital injected into one brachial vein. The kidneys, the lungs and the liver were then ully removed In some expts. heart, spleen, adrenal glands, piece of thigh muscle, some blood and t of the intestine were also removed. The ipsilateral kidney as divided into cranial, middle and caudal ts, each roughly corresponding to division (Fig. 1) and the weight of each part was determined. The was divided into several parts. A two-channel spectrometer was used to analyse the samples. Propor onality was present in the range of isotope activity used The dual-isotope technique allowed simulta- ous determinations of either 57-Co and 58-Co or 57-C and 85-Sr isotopes. The hardness of the gamma- mission made correction for absorption in the samples unnecessary

Calculation of recovery Isotope activity exceeding 1 of the injected activity as found only in the dneys, the lungs and the liver The sum of these activities was therefore regarded as the *total recovery* he recovery of Co nuclides (large spheres) averaged 93.2 % ± 5.7 (S D) of the injected activity Recovery isotope activity in an organ was always expressed as percentage of the total recovery Ideally these lues should represent the fraction of injected microspheres trapped in the respectiv organ

alculation of the apparent tubular excretion fraction (ATEF)

fter injection of substance into leg vein the ATEF of this substance was calculated by dividing the ount excreted in the urine on the side of the injection with the amount jected (Sperber 1948 b)

Ligation of shunts in the renal portal system

Anaesthesia as induced ith 20–30 mg/kg sodium pentobarbital The right kidney was exposed after flank incision. In some hens the renal anastomosis was partly freed from the ventral surface of the kidney by blunt dissection and ligated close to the bifurcation of he external iliac vein (ligature 1 Fig. 1). This ligature should prevent renal portal blood from perfusing the cranial part of the kidney and from being shunted to the lungs via the tw cranial shunts (Akester 1967) In other animals, an additional ligature was placed around the caudal branch of the renal portal vein, here this vessel leaves the kidney (ligature 2, Fig. 1) The tw ligatures should force all venous blood from the leg to perfuse the peritubular capillaries of the middle and caudal parts of the kidney (Akester 1967).

Fluorescein angiography

In 13 hens the right kidney was exposed as described bove and illuminated by 400 W metallic halogen lamp (HQI Osram GMB, München, West-Germany) Fluorescein sodium (40 solution) as then

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The blood flow in the renal portal system of intact hens was characterized by determining the fractional distribution of 15 µm and 50 µm microspheres in this system after injection into leg veins. Validation showed that only 50 µm spheres gave a reliable estimation of this distribution. The blood flow in the coccygeomesenteric vein was directed towards the liver in nearly all cases. On the average 44, 47 and 1 % of the portal blood from the external iliac vein perfused the ipsilateral kidney, the liver and the lungs, respectively. However, the distribution of portal blood to these organs varied considerably between individuals and changed appreciably within 35 min in half the animals studied. The reason for these variations is not clear. The portal blood from the right and left external iliac veins was asymmetrically distributed in most cases, illustrating the importance of local factors in its regulation. Variation was great in the regional distribution of portal blood within the kidney possibly due to local vasoconstriction of portal vessels. Further information on the regulation of renal portal blood flow is needed to explain its physiological significance. Microspheres provide a convenient method for such studies. A combination of microsphere and Sperber techniques allows us to determine the renal excretion efficiency of a given substance.

Key words: Portal system, microspheres, birds, kidney, phlebography, venous circulation

The avian kidney is supplied by blood from the arterial and the renal portal circulations (Sperber 1948 a, Fig. 1). The physiological significance of blood flow in different parts of the renal portal system and its regulation is not fully understood (Gilbert 1961, Sillér 1971). The dual blood supply to the avian kidney has been used extensively to study renal handling and effects of drugs, hormones and auto-pharmacological agents (May and Carter 1970, Bond 1973, Rennick 1976) utilizing a technique developed by Sperber (Sperber 1946, 1948 a, b). There is, however, an unpredictable variation in individual results obtained with the Sperber technique (Sperber 1948 b) which might be related to variations in blood flow in the renal

This work was presented in part at the XV Scandinavian Congress of Physiology and Pharmacology in Aarhus, Denmark, Aug. 1976 and published in abstract form (Acta physiol. ... Suppl. 440, 190.

system (Akester 1964). The aim of the present investigation was to get quantitative ...ation about the distribution of blood flow in the renal portal system, under physiolo- ...onditions and in connection with the Sperber technique. Since the microsphere tech- ...as chosen for this purpose, it was also necessary to test the validity of the technique applied to a venous circulatory system.

Materials and Methods

...White Leghorn hens, 6–18 months of age and weighing between 1.1 and 1.4 kg, from 5 different ...d deries and one lot of non-laying White Leghorn pullets, 4–5 months of age and weighing between ...d 1.1 kg were used. The animals were kept on commercial feed for laying hens (Pulfor® Lant-..., Sweden) but were fasted over night before the expts. They had free access to tap water.

...sphere procedures

...microspheres of 15 ± 5 μm diameter (mean ± S.D. 3 M Co. St. Paul, Minnesota, U.S.A.), labelled 85-Sr and 3 batches of Tracer Sephadex microspheres (Pharmacia Fine Chemicals AB, Sweden) of 15 μm diameter (mean ± S.D.) were used. The large spheres (50 μm) were labelled with 57-Co or ... by ... procedure recommended by the manufacturer and were carefully washed with distilled water and for more than ... week. The small spheres (15 μm) were subjected to homogenization shortly before ...ection in order to break up possible aggregates. The spheres were suspended in less than 1 ml of ...solution of Ficoll 70 (Pharmacia Fine Chemicals AB, Sweden) or in chicken plasma and were agitated ...ously prior to injection. The suspensions were injected at ... rate of 0 ... –0.4 ml/min, followed by ... slow ...tion of 0.5 ml saline. Animals were sacrificed 5 (3–5), 25 or 60 min after the injection, using 75–100 mg/...of sodium pentobarbital injected into one brachial vein. The kidneys, the lungs and the liver were then ...ly removed. I... some expts. heart, spleen, adrenal glands, ... piece of a thigh muscle, some blood and ...t of the intestine were also removed. The ipsilateral kidney was divided into cranial, middle and caudal ...s, each roughly corresponding to ... division (Fig. 1) and the weight of each part was determined. The ... was divided into several parts. A two-channel spectrometer was used to analyse the samples. Propor...ality was present in the range of isotope activity used. The dual-isotope technique allowed simulta...us determinations of either 57-Co and 58-Co or 57-Co and 85-Sr isotopes. The hardness of the gamma-...ation made correction for absorption in the samples unnecessary.

Calculation of recovery. Isotope activity exceeding 1% of the injected activity was found only in the ...kidneys, the lungs and the liver. The sum of these activities was therefore regarded as the *total recovery*. ...e recovery of Co nuclides (large spheres) averaged 93.2% ± 5.7 (S.D.) of the injected activity. Recovery ...isotope activity in an organ was always expressed as ... percentage of the total recovery. Ideally these ...ues should represent the fraction of injected microspheres trapped in the respective organ.

...ulation of the apparent tubular excretion fraction (ATEF)

...fter injection of ... substance into ... leg vein the ATEF of this substance was calculated by dividing the ...ount excreted in the urine on the side of the injection with the amount injected (Sperber 1948 b).

Ligation of shunts in the renal portal system

Anesthesia was induced with 20–30 mg/kg sodium pentobarbital. The right kidney was exposed after ... flank incision. In some hens the renal anastomoses was partly freed from the ventral surface of the kidney by blunt dissection and ligated close to the bifurcation of the external iliac vein (ligature 1, Fig. 1). This ligature should prevent renal portal blood from perfusing the cranial part of the kidney and from being shunted to the lungs via the two cranial shunts (Akester 1967). In other animals, an additional ligature was placed around the caudal branch of the renal portal vein, where this vessel leaves the kidney (ligature 2, Fig. 1). The two ligatures should force all venous blood from the leg to perfuse the peritubular capillaries of the middle and caudal parts of the kidney (Akester 1967).

Fluorescein angiography

In 15 hens the right kidney was exposed as described above and illuminated by ... 400 W metallic halogen lamp (HQY ... Osram GMB, München, West-Germany). Fluorescein sodium (40 ... solution) was then

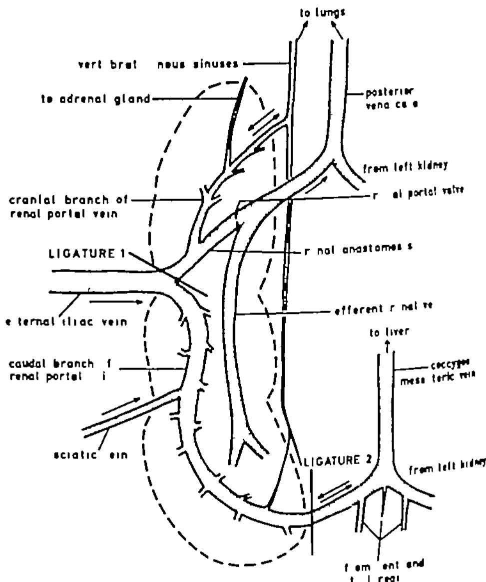


Fig 1 Principal veins of the renal portal system of the hen (right kidney seen from the abdominal ca... Venous blood from the majo part of the leg enters the ipsilateral renal portal vein (*iliaca interna/iliac* renal vein) via the external liac vein (*iliaca xterna*). The sciatic vei (*v ischiadica*) conveys ... from the caudal thigh region to the caudal branch of the renal portal vein. The cranial branch of the re... portal vein communicates w th the vert bral venous sinuses (Akester 1967) and with the adrenal po... system (Sperber 1948). The caudal branch of the renal portal vein communicates with the correspond... vessel from the other kidney with veins (*i.e.* *coccygea*) draini g the vent and tail regions and with ... large coccygeomesenteric vei (*coccygo-mesenterica*) draini g the hind gut. The last vessel establishe... link between the renal and the hepatic portal systems. The caudal branch of the renal portal vein is ... also co nected with the vertebral venous nuses (Sperber 1948a, Burger and Estavillo 1977) The re... portal vein branches into the cranial, middle and caudal divisions (Goodchild 1956) of the kidney ... terminates in the peritubular (ntert bular) network of capillary sinuses, where it mixes ith the p... glomerular (arterial) blood (Sperber 1948a) Perit bula blood eventually leaves the kidney by the effer... renal vein (*r renalis magna sive revehens*) which is connected to the external iliac vein by the renal a... stomosis. The latter vessel has sphincter—the renal portal valve. When this sphincter is relaxed, bl... in the portal system can bypass the kidney tubules and go directly t the lungs via the posterior ena ca... Thus, there are 4 possible majo afferent routes (rrows) t the renal portal system and at least 3 po... bilities for the blood in this system to bypass the kidney (arrows). Ligatures 1 and 2 (see text) are pla... around vessels at points indicated by crossmarks.

into leg vein at the same rate as used in the microsphere injections. The kidney was observed barrier filter (see below). In 5 hens these events were recorded photographically by serially re-exposures, (2 pictures per using Nikon F camera with MD 2 motor (Nikon Co, Japan). The Ektachrome EH 135 (Eastman Kodak Co, U.S.A.). An excitation filter (Schott BG 3) was mounted of the lamp and barrier filter (Schott OG 495) was fitted to the camera lens.

General procedures

Experiments, the hens were unanaesthetized and sitting in their normal perching position except following operative procedures and during fluorescein venography when they were anaesthetized and supine. They were only restrained in harness of cloth (Campbell 1960). The animals appeared to be fully at ease. Urine was collected through two plastic funnels sewn one over each ureteral opening according to modification (Campbell 1960) of Sperber's original technique (Sperber 1946, 1948). Each funnel was perfused with distilled water by an infusion pump at rate of 1.0 ml/min. Urine was collected in plastic vials on collector. Injections were made through polyethylene catheters (PP 25 or PP 50). Microspheres, p-aminohippuric acid (PAH) and fluorescein sodium were injected through catheter placed in distal vein of the leg, usually the medial tarsal vein, and always on the right side, unless stated otherwise. PAH—mg per injection—was administered as the sodium salt (Merck Sharp and Dohme Co., U.S.A.) at infusion rate of 0.6–1.4 mg/kg/min and determined in the diluted urine according to Brun (1951).

To investigate whether the microsphere injections had functional effects on the kidney glomerular filtration rate and renal clearance of 125-I-Na-o-iodohippurate (Kabi Diagnostika, Stockholm, Sweden) were measured before and after 2 doses of microspheres. For the clearance measurements, tracer doses of EDTA (ethylenediamine-tetraacetate; Behringwerke AG, Marburg, West Germany: 3 10^4 cpm/ml) and 125-I-Na-o-iodohippurate (6 10^4 cpm/ml) were infused through catheter placed in one brachial vein of 3 animals, respectively. In one hen the infusion was made through catheter in leg vein on the side, which allowed ATEF to be calculated. After priming dose of 0.75 ml the infusion rate was ml min. At least 2.5 h of infusion were allowed to pass before starting the clearance determinations. Blood samples were drawn in the middle of each 10 min clearance period from catheter placed in vein of the left leg and centrifuged immediately. Isotope activity was then determined in 0.5–0.6 ml of plasma and the urine samples. After 2 or 3 clearance periods during steady state, consecutive injections of 0–15 000 large spheres, at 35 min interval, were made into leg vein on the right side as described in relation to Fig. 5. Each injection was followed by 2 or 3 clearance periods. Clearance values were calculated for each kidney separately as the mean of values from 2 or 3 collection periods. No correction for the protein binding of the test substances was made.

Statistical analysis

Results are given as the arithmetic means ± standard deviation (S.D.) or means ± standard error of the mean (S.E.) when appropriate. Differences in recovery of microspheres were analysed statistically with *t* for paired observations (Armitage 1973) and these differences were considered significant at the 0.05 level. The orthogonal regression coefficients (Geigy Scientific Tables 1970) were used to calculate the regression functions.

Results

Validation experiments

Suitable size of spheres. 1 1/2 h after injection into brachial vein the recovery of small and large spheres in the lungs was 98.6% (3 hens) and 98.5% (5 hens), respectively. The escape of both types of spheres to the arterial circulation of the hen was therefore negligible, as previously reported for 15 μm spheres (Boelkins *et al.* 1973). After injection into the coccygeomesenteric vein (Fig. 1) ligated caudal to the point of injection, 99.5% of the large (4 hens) and 99.7% of the small (2 hens) spheres were recovered in the liver 1 1/2 h post-injection. Thus both types of spheres were effectively trapped in the liver. After the simultaneous injection of both types of spheres into a leg vein of non-ligated hens, the recovery of the large spheres was on the average 1.37 ($p < 0.001$) and 0.51 ($p < 0.001$) times that of the small

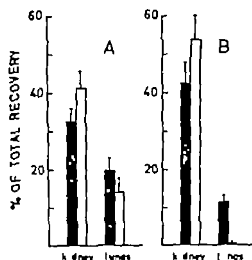


Fig. 2. Unequal distribution of small and larg
Recovery of 15 µm (■) and 50 µm (□) sphe
silateral kidney and lungs, after simultaneous
into a leg vein of A) 21 non-ligated hens and
ligation of major shunts to the lungs in 5 hen
are given as mean values (columns) and 1 S.E.
lines). Asterisks indicate statistically significa
ences in mean recoveries of small and large sp

spheres in the *ipsilateral kidney* and lungs, respectively (Fig. 2 A). These differ
creased with time for the three groups of hens sacrificed 5, 25 or 60 min post-injectic
the renal anastomosis was ligated (ligature 1 in Fig. 1) the large spheres—in sharp
to the small ones ($p < 0.001$)—were almost completely prevented from reaching
(Fig. 2 B). On the other hand, the fraction of large spheres trapped in the ipsilater
of these hens was 1.28 times ($p < 0.01$) that of the value for the small ones. In th
animals where the shunts both to the lungs and the liver had been blocked (ligatur
in Fig. 1) 99.5% of the large spheres were recovered in the ipsilateral kidney 1 I
injection. These findings indicate that only the large spheres were effectively trap
peritubular capillaries of the kidney. This is consistent with the finding that wher
hippuric acid (PAH) and the small spheres were injected together $ATEF_{PAH}$ often
the fraction of spheres recovered in the ipsilateral kidney (unpublished observation
the larger spheres were consistently found in excess (see below). In view of all the
the large spheres were chosen for further expts.

Random errors of counting and distribution. The dosage of spheres administe
minimum counting precision of ±1.0 per cent (standard deviation as percentage
count) for all tissues where recovery was more than 5% of total recovery. Buck
(1971) concluded theoretically and experimentally that the difference between t
trations of spheres in two random samples is usually less than 20% provided the
ber of spheres in the samples is greater than 400 and only random factors affect th
tion. In the present study 7 500 to 15 000 of the large spheres were injected an
400 microspheres per organ correspond to approximately 3 to 6% of the total rec
combined experimental variability was assessed (Neutze *et al.* 1968) by injecting
labelled spheres simultaneously (Fig. 3). Organ recovery of 58-Co was taken a
and the deviations of the recovery of 57-Co from this reference was calculated
centage. The standard deviation of these differences in recovery of the two nuclid
than 5% for organs with a high mean recovery *i.e.* ipsilateral kidney and liver a
13 to 19% for organs with a low mean recovery *i.e.* lungs a — ateral ki

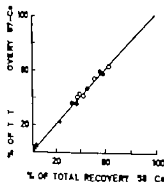


Fig 3

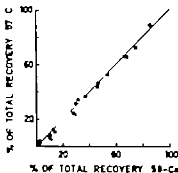


Fig. 4

Comparison of dual-isotope determinations. Recovery of 57-Co and 58-Co labelled spheres in al kidney (●), contralateral kidney (Δ), lungs (+) and liver (○) in 5 hens after simultaneous n into leg vein In some animals the recovery of each isotope was below 1.5 % in lungs or con- al kidney and these values were excluded. Line of identity is drawn

Sphere distribution after separate injections. Recovery of 50 µm spheres in the ipsilateral kidney (●), teral kidney (Δ), lungs (+) and liver (○) in 11 hens Spheres, labelled with either 57-Co or 58-Co, jected simultaneously but through separate catheters, placed at different levels of one vein (= 5) o different veins (n 6) of the leg. In some animals the recovery of each isotope was below 2 in r contralateral kidney and these values were excluded. Line of identity is drawn. The regression s are y 1 14x–2 27 and y = 1 06x–4 62 for the ipsilateral kidney and the liver values respectively rresponding linear correlation coefficients ( ) are = 0 95 and = 0 97 respectively

f precision for the low recoveries is however of minor importance in the present study e fractional distribution was determined.

gree of mixing of spheres with venous blood from the leg In 5 hens, two catheters were d in the same distal vein of the leg, but through separate incisions and with the tip of theters 2 to 3 cm apart. In 6 other hens the two catheters were inserted into the medial teral tarsal veins, respectively Spheres, labelled with either 57-Co or 58-Co were then ed simultaneously through the separate catheters. In both types of experiments the distribution of the two nuclides was similar (Fig. 4), indicating acceptable mixing of es with the blood from the lower leg.

orescein injections. Injections of fluorescein sodium into the leg vein gave an intense ish-green fluorescence, first seen in the major portal vessels and then in the small s on the renal surface After 20–30 s it had reached other organs, via the arterial cir ion. At this time, the vessel walls were also fluorescent, which obscured the intraluminal escence. In the early phase, however the location of the fluorescence in the portal ls could be evaluated, especially with the rapid-sequence venography Fluorescence was in the renal anastomosis in all hens, and in some cases also in the caudal branch of the portal vein. After most injections stream-lining of the fluorescence was seen in the r portal vessels. Location of the fluorescence within a vessel often fluctuated (within nds), in some cases with the phases of respiration. This is in agreement with observations e flow in comparable veins of several mammalian species (Helps and McDonald 1954). ever, in the present context it is important to note that there was no preferential location he fluorescence in the external iliac vein.

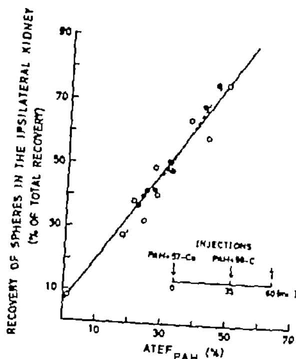


Fig. 5. Comparison of tubular e... PAH ($ATEF_{PAH}$) and recovery of ... the ipsilateral kidney 57-Co and 85-... spheres were injected together with ... a leg vein of 9 hens 60 min (●) and ... respectively prior to sacrificing ... (see inserted Fig.). In 2 other hens ... injection was made. In 2 others only ... one. The regression lines for the ... 1.48x + 5.20 solid) and the 25 min ... broken) values are drawn. Linear ... coefficient (r) was r 0.96 and r=0... 60 min and the 25 min values, respect...

Comparison of techniques of microsphere recovery and PAH excretion for the esti... portal blood flow. The relationship of ATEF for PAH ($ATEF_{PAH}$) and recovery of s... the ipsilateral kidney was studied by injecting labelled spheres together with PAH. T... schedule as described in the legend to Fig. 5 was chosen because ipsilateral excess ... excretion was observed only during the first 20 min after an injection. Total reco... PAH in the urine averaged 87% after both injections. $ATEF_{PAH}$ is proportional ... fraction of venous plasma flow from the leg that perfuses the ipsilateral kidney wh... certain conditions are fulfilled. Firstly the peritubular plasma concentration of PAH ... in this study must have been well below that needed to saturate the renal transport c... of this substance (Tm_{PAH} Dantzler 1966, Svendsen and Skadhauge 1976 and Odlind ... lished). Secondly the excess of PAH excreted on the side of injection must originate excl... from extraction of the substance during first passage through the ipsilateral kidney. G... ular filtration rate and total renal plasma flow were not determined and corrections ... influence of possible asymmetries in these respects on ATEF could not be made. This ... contribute to the scatter of values observed in Fig. 5.

The ATEF values of PAH were found to be closely related to the recovery of sphe... the ipsilateral kidney (Fig. 5). This indicates that the spheres and PAH were similarl... tributed to the peritubular capillaries of the ipsilateral kidney. The dual-isotope tech... allowed this relationship to be studied at 2 different times, as described in the lege... Fig. 5.

Effect of injections of spheres on renal function. Data are given in Table I. Two consec... injections of spheres caused no impairment of the renal clearance of 51-Cr EDTA. ... implies that glomerular filtration rate was unaffected since the renal clearance of 5... EDTA equals that of 3-H inulin in the hen (Odlind 1976). There were no statistically

Effects of two consecutive injections of spheres on renal function. For details see Experimental procedures. Clearance values (mean values in ml/min/kg) for 51-Cr EDTA (C_{EDTA} 5 hens) and 125-I-Na-o-iodohippurate (C_{Hipp}, 3 hens) and ATEF values of the latter ($ATEF_{Hipp}$, 1 hen) are shown. The paired differences between ipsilateral and contralateral clearance values are given as means ± S.D. Recovery of spheres in the ipsilateral kidney was 42–83 ...

	ipsilateral kidney	contralateral kidney	difference (ips.-cont.)	
inj	2.12	2.07	0.05±0.14	
st inj	2.05	2.13	−0.08±0.09	
nd inj	2.11	2.13	−0.02±0.07	
				$ATEF_{Hipp}$
inj	19.6	18.3	1.3±0.8	44.9
1st inj	17.1	18.5	−1.4±0.8	41.3
2nd inj	16.9	18.5	−1.6±1.1	40.1

...two injections of spheres. In another hen the two injections of spheres caused no change ... ATEF values of continuously infused 125-I Na-o-iodohippurate. These findings suggest ...he injections of spheres had no effect on the tubular secretion of the acid. Furthermore, ...e change in total renal plasma flow could not have occurred without a change in C_{Hipp}. ...over the ratio of ATEF $_{AH}$ to recovery of spheres in the ipsilateral kidney (calculated ...data in Fig. 5) was similar (p 0.05) after the first (60.1 ±4.5% mean ± S.D.) and the ...nd (65.1±6.9%) injection. This shows that the excretion efficiency of PAH was un...ted by a preceding injection of spheres.

...Distribution of spheres in different tissues

...ue distribution. Fig. 6 shows the fractional distribution of spheres in 76 intact hens of five ...treat lots and in 13 hens prepared for urine collection according to Sperber. A high re...ry in one organ of a given animal, is accompanied by a correspondingly lower uptake ...the other organs. Recoveries were determined at 5 or 25 or/and 60 min after the injection ...to ...hether there was any redistribution of the spheres. In intact as well as prepared hens ...re were no statistical differences in mean recovery of spheres in the respective organs at ...three time intervals post-injection. This indicates that redistribution of spheres (50 µm) ...ing the first hour after the injection was negligible.

...Also, there ...as no statistical difference in mean recovery of spheres between intact and ...pared hens which indicates that the latter procedure did not cause any important changes ...portal blood flow distribution compared to the intact animal.

...Taking all animals together (102 injections in 89 animals) the recovery of spheres in the ...ilateral kidney averaged 43.5±17.8% (S.D.). Hepatic recovery averaged 47.3±20.6% ...D.) and was less than 5% in only 5% of the injections. Pulmonary recovery averaged ...%, and exceeded 10% in less than 22% of the injections. Recovery in the contralateral ...ey averaged 1.5% and exceeded 5% in only 6% of the injections. The inter-individual ...iation in tissue distribution was extensive also within a single lot, as exemplified by Fig. 7

...Sphere distribution within the ipsilateral kidney. Spheres were unequally distributed in the ...erent parts of the kidney. In 84 hens the distribution of spheres within the ipsilateral kidney

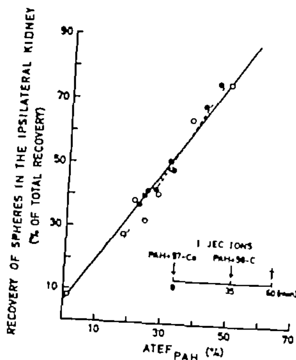
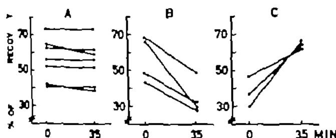


Fig. 5 Comparison of tubular PAH ($ATEF_{PAH}$) and recovery of the ipsilateral kidney 57-Co and 58 spheres were injected together ... a leg vein of 9 hens 60 min (●) and respectively prior to sacrificing (see inserted Fig). In 2 other hens injection was made. In 2 others only one. The regression lines for the 6 1 48 + 5.20 (solid) and the 25 min ... broken) ... are dra n. Linear coefficient ( ) was r = 0 96 and r = ... 60 min and the 25 min values, respect...

Comparison of techniques of microsphere recovery and PAH excretion for the est... portal blood flow The relationship of ATEF for PAH ($ATEF_{PAH}$) and recovery of ... the ipsilateral kidney was studied by injecting labelled spheres together with PAH. ... schedule as described in the legend to Fig. 5 was chosen because ipsilateral exces... excretion was observed only during the first 20 min after an injection. Total rec... PAH in the urine averaged 87% after both injections. $ATEF_{PAH}$ is proportiona... fraction of venous plasma flow from the leg that perfuses the ipsilateral kidney ... certain conditions are fulfilled. Firstly the peritubular plasma concentration of PAH in this study must have been well below that needed to saturate the renal transport ... of this substance (Tm_{PAH} Dantzler 1966, Svendsen and Skadhauge 1976 and Odlind ... lished) Secondly the excess of PAH excreted on the side of injection must originate exc... from extraction of the substance during first passage through the ipsilateral kidney ... ular filtration rate and total renal plasma flow were not determined and corrections ... influence of possible asymmetries in these respects on ATEF could not be made. The... contribute to the scatter of values observed in Fig. 5

The ATEF values of PAH were found to be closely related to the recovery of sph... the ipsilateral kidney (Fig. 5) This indicates that the spheres and PAH were similar... tributed to the peritubular capillaries of the ipsilateral kidney The dual-isotope tech... allowed this relationship to be studied at 2 different times, as described in the leg... Fig. 5

Effect of injections of spheres on renal function Data are given in Table I. Two conse... injections of spheres caused no impairment of the renal clearance of 51-Cr-EDTA ... implies that glomerular filtration rate was unaffected since the renal clearance of 5... EDTA equals that of 3-H-inulin in the hen (Odlind 1976). There were no statistically ... nificant changes in bilateral renal clearances of 125-I Na-o-io... ($C_{...}$) follo...



A–C. Change in renal uptake of spheres with time. Recovery of 50 µm spheres in the ipsilateral after two consecutive injections at 35 min interval, using the dual-isotope technique. The spheres injected through catheter into leg vein of the right leg of 14 hens. Results are given in three groups: changed, B) decreased and C) increased renal uptake of spheres. Note broken scale on the ordinates. connect the two observations of each animal.

high, however uptake in the caudal part of the kidney dominated, and only a few res were found in the cranial part. A similar skew distribution within the kidney was times seen for radiopaque fluid when injected into a leg vein (unpublished observa-)

Asymmetric distribution after bilateral injection Using the dual-isotope technique, spheres injected simultaneously into a vein of the right and left legs, respectively. In most hens, as well as non-laying, spheres injected on the right side showed a different organ ribution compared to spheres injected on the left side. This is illustrated in Fig. 7 for the eries in the ipsilateral kidney and the liver but was also found for the recoveries in the s. For spheres injected on the right side (18 hens), the average recovery was 26.2% lower (1) in the kidney and 26.4% higher (p 0.05) in the liver compared to the corresponding results for spheres injected on the left side. Laying and non-laying hens did not in this respect. The average recovery in the lungs was the same regardless of the side of ction.

Change of distribution with time These expts. were designed to see if the distribution of eres to the organs changed with time or as the result of a previous injection. Two suspensions of differently labelled spheres were injected with a 35 min interval (as described in legend to Fig. 5). There was no difference in the mean recoveries of the two nuclides; the ipsilateral kidney it was 49.9 ± 4.1% (S.E.) and 49.0 ± 3.2% (S.E., p > 0.8) after the and the second injection, respectively in 14 animals. (From data in Fig. 8 A–C.) Thus, re was no clear effect of the first injection of spheres on the distribution after the second . However the distribution of spheres after 2 injections could vary considerably in the dividual hen, as is shown in Fig. 8 B and C for the ipsilateral renal recovery in seven hens. 7 other hens the two nuclides were equally distributed (Fig. 8 A).

Discussion

Validity of the microsphere technique in the study of the renal portal circulation

The 15 µm microspheres could not be used, since they were incompletely trapped in the intertubular capillaries of the kidney (Fig. 2), in contrast to the almost complete pulmonary

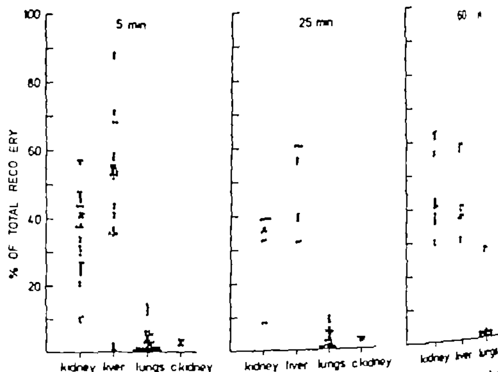


Fig. 6. Distribution of spheres i different organs. Organ recoveries of 50 μm spheres injected ... of the right leg i 1 76 intact hens (●) at 5 (n = 46), 25 (n = 15) or 60 min (n = 15) respectively and ... prepared for urine collection according to Sperber (○) at 25 and 60 min, post-injection. Values ... the contralateral kidney (c. kidney) are not shown. L kidney stand for ipsilateral kidney. The ... of injections are given.

(mean ± S E.) was 14 2 ± 1 6 %, 40.9 ± 1 9% and 45 0 ± 2.1 % in the cranial, mi... caudal parts, respectively The weight (mean ± S.E.) of these parts were 2.49 ... 2.21 ± 0 08 g and 3 11 ± 0 11 g, respectively for 48 kidneys. In animals with a low ... recovery the spheres were evenly distributed between the three parts. When hepatic ...

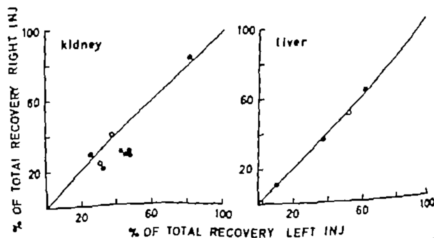


Fig. 7 Asymmetric distribution of spheres after bilateral jection. Recovery of 50 μm pheres i... kidney and liver after simultaneous injection nt veins of the right (ordinates) and the left (abs... of 12 laying hens of the same lot (●) and 6 non-laying (○) hens of diff ... t, using the d... technique. Asterisks indicate mean values. Lines of identity are drawn

...n of blood flow in the renal portal system

...d flow in the coccygeomesenteric vein was nearly always directed towards the liver ...re were spheres in this organ after 95% of the injections. This observation is sup... ...y the high and persistent recovery of spheres in the caudal part of the kidney A flow ...ection of the liver is in agreement with the findings of Sturkie and Abati (1975), ...g as well as non-fasting birds, using electromagnetic flowmeter probes. Other work ...e reported a blood flow in both directions between the two portal systems (Spanner ..., Clarkson and Richards 1966, Akester 1967 Purton 1970, 1975). These studies, ...r include different experimental procedures such as anesthesia, supine position or ...nal surgery which could have affected the hemodynamic situation in the portal ...), as pointed out by Sperber (1948 a). Results presented in the present paper are ...onscious hens in a normal body position, and are therefore presumably more ...ntative of conditions in the intact animal.

...more difficult to estimate the direction of flow in the most cranial shunt (Fig. 1), ...erall recovery of spheres in this part of the kidney was low The small size of this ...however makes a large contribution by backward" flow from the vertebral sinuses ...able (Akester 1967). Regional differences in the intrarenal distribution of spheres ...e due to local vasoconstriction of major portal vessels in the kidney as proposed by ...r (1967). That local ...asoconstriction can occur in the renal portal system is suggested ...radiographic studies on the renal localization of 35-S-furosemide when injected into ...ea. The distribution of this diuretic was often restricted to certain regions, leaving ...t areas unlabelled (Dencker and Odlund 1977). A nervous regulation of blood flow ...renal portal system is implied by the dense, predominantly adrenergic innervation of ...els (Bennet and Malmfors 1970).

...mmary it is obvious that in the unanesthetized hen the bulk of the blood in the ...portal system is supplied by the external iliac and the sciatic veins. The blood from ...atic vein could either have perfused the caudal part of the kidney or could have been ...d along the coccygeomesenteric vein to the liver Spheres were not injected into the ... vein since it is inaccessible to external catheterisation However due to the larger ...f the external iliac vein the fractional distribution of blood flow from this vessel should ...t that of the renal portal system as a whole.

...bution of blood flow in the renal portal system

...resent study demonstrates a striking variation in the distribution of renal portal blood ...between individuals (Fig. 6), within the kidney between right and left side (Fig. 7) and ...time (Fig. 8).

...ken together these results reveal a very complex flow pattern in agreement with Akes... (1964 1967) findings in anesthetized birds using a radiographic (qualitative) technique. ...e general conclusions are of interest. On the average, the kidney received less than half ...e portal blood from the leg, and in no case did it receive all of this blood. Thus, shunt ...ie portal blood bypassing the kidney tissue, was always present. In nearly all hens the ...)geomesenteric vein was the dominant shunt vessel. This fits with the high rate of blood ...in this vessel as reported by Sturkie and Abati (1976). One could deduce from these

...ec)

and hepatic extraction of these spheres. This difference suggests a "leakage" of some of the small spheres initially deposited in the kidney and may be a consequence of the [illegible] structure of the peritubular capillaries in the renal cortex of birds (Sperber 1948 a). Extra[illegible] pulmonary extraction of both types of spheres excludes recirculation of any importance. It [illegible] crucial, when using microspheres to assess blood flow distribution, that the spheres [illegible] adequately mixed with the blood and distributed to various organs in proportion to the blood flow In the arterial circulation mixing can be accomplished by injecting the spheres into the left heart ventricle (Hales 1974) and can be verified by comparing a) the [illegible] spheres in paired organs, *i e* kidneys (Neutze *et al* 1968 Boelkins *et al* 1973) or b) the con[illegible] centration of spheres in peripheral arteries (Neutze *et al* 1968, Hoffbrand and Forsyth [illegible] In analogy with these results, in the present study spheres reaching the right ventricle, [illegible] found to be equally distributed between the two lungs ($y = -0.08 + 1.06X$ linear correlation coefficient $r = 0.98$) However mixing conditions in the peripheral venous circulation [illegible] not easily evaluated Nolan *et al* (1975) found nonhomogenous distribution within the liver of colloidal $Cr^{32}PO$ or 15 μm resin microspheres after injection into the portal vein [illegible] rats, in contrast to the homogenous dispersion in the liver of $Cr^{32}PO$ injected into an [illegible] artery The difference was attributed to stream-lining of the particles in the blood [illegible] portal injection In the present study the spheres were injected into a distal vein at a [illegible] rate, which resulted in reasonably even mixing of the spheres with the blood from the [illegible] leg (Fig. 4) However the application of spheres to the study of the venous circulation [illegible] sents another validation problem since venous blood is continuously being added by [illegible] proximal to the site of injection This could result in a preferential peripheral streaming [illegible] the spheres in the main vein and hence would lead to a systematic distributional error [illegible] bifurcations. It was therefore essential to see if this occurred at the most critical point, [illegible] is in the external iliac vein It was not possible to visualize microspheres labelled with fluores[illegible] cein-isothiocyanate in the larger vessels, possibly due to axial streaming (Phibbs and Do[illegible] 1970) and coating of the spheres with erythrocytes (Phibbs *et al.* 1967). However there [illegible] no preferential streaming of fluorescein in the external iliac vein and it seems likely [illegible] PAH a substance of comparable molecular weight, would be handled similarly Since [illegible] was shown that the spheres and PAH were similarly distributed to the peritubular capillaries of the ipsilateral kidney (Fig. 5) it seems reasonable to exclude preferential peripheral streaming of spheres in the major portal vessels as an important source of error in the overall distribution Possible distributional errors due to fluctuations between axial [illegible] peripheral streamings were minimized by making the injections slowly for a period of 2 [illegible] 4 min

It seems unlikely that gravitational forces could influence the distribution of spheres [illegible] the relative density of Tracer Sephadex (large) spheres is close to that of red blood cells [illegible] 1 12 g/ml and 1 10 g/ml respectively From the validation experiments with 40 [illegible] microspheres, it can be concluded that the technique used, gives a reliable estimation of [illegible] fractional distribution of blood flow from the external iliac vein to the kidneys, the lungs and the liver

the coccygeomesenteric vein and therefore also the hepatic portal system are parts. reduces the role often ascribed to the renal portal valve as the dominant regulatory of portal blood flow to the kidney (Rennick and Ghandia 1954, Gilbert 1961). The sphere technique provides a valuable tool for further studies of the regulation of renal l blood flow in the hen.

... work ... as supported by grant no. 2874 from the S edish Medical Research Council. I am ...ted to Mrs Sigrid Sandberg for skilful technical assistance.

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authors findings that fasting affects the distribution of renal portal blood flow The possibility remains, however to be tested Spheres terminating in the lungs could have bypassed the kidney *via* one or both of the cranial shunts (Fig. 1) and the technique used cannot discriminate between these routes. However the renal portal valve must have been closed or nearly closed in the majority of cases (Fig. 6) Contrary to these findings in intact birds the most common shunting route in supine and anesthetized birds was that through the renal portal valve (Akester 1967) It is obvious, that the renal portal valve is only one of several factors involved in the regulation of renal portal blood flow

Some further points are worthy of note. In most animals, blood flow from the right and left external iliac veins was differently distributed (Fig. 7). This is in agreement with the findings that radiopaque fluid took different routes through right or left renal portal system after bilateral injections in about 25 of the birds (Akester 1967).

The reason for a higher average flow to the kidney from the left external iliac vein compared to the right one (Fig. 7) is not clear The difference was not due to the large size of the oviduct attached to the left kidney since it was seen in laying as well as non-laying hens

Duplicate determinations, at 35 min interval revealed a considerable change in the pattern from the leg in 50 % of the hens, in spite of constant experimental and environmental conditions. There was a covariation in these cases mainly between the renal and the femoral fractions. The physiological basis for this "intrinsic" variation with time is not clear and merits further studies. Very rapid (within seconds) vascular readjustments, related to the renal portal valve have been described by Akester (1964). Interestingly the valve has a dense autonomic innervation (Gilbert 1961 Akester and Mann 1969 Dolezel and Zahradka 1969 Bennet and Malmfors 1970) which has been studied in vitro by Rennick and Gandia (1954) and by Bennet and Malmfors (1975).

The fraction of portal out of total blood flow to the kidney is difficult to measure directly It has been suggested to be 2/3 (Sperber 1960) or 1/2 (Skadhauge 1973) in conscious birds, ratios calculated from indirect observations. Assuming a relatively constant venous return from the resting leg, it may be expected that the large intra- and interindividual variations in the renal fraction of portal blood described in the present paper reflect a variation in renal perfusion rate. This would lead to highly variable renal portal to total blood flow ratios and is of considerable interest since it provides a possible mechanism by which total peritubular blood flow can change irrespective of the arterial blood flow to the kidney. In this connection, it is worthy of note that Sapirstein and Hartman (1959) found a considerably higher arterial perfusion rate of leg muscles than of pectoral muscles in the anesthetized bird, providing a high venous return from the leg and thus to the renal portal system.

It is evident (Fig. 5 and 7) that the individual results obtained with the Sperber technique will vary mainly with the renal portal perfusion fraction A combination of the microsphere and the Sperber techniques, however enables a correction for the influence of "shunting" of renal portal blood on the excretion (ATEF) values. This will also allow an estimation of the renal excretion efficiency that is the true tubular excretion fraction (Sperber 1948 b) of a given substance.

In sum, it seems that changes in the blood flow distribution in the renal portal system are the effect of hemodynamic readjustments (sometimes rapid) in a [illegible] network, in

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From the Department of Neurology University of Helsinki, Finland

Effects of pindolol, sotalol and the optical isomers of propranolol on muscle action potentials and depolarization-secretion coupling in the rat

By

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Abstract

...SEN, A. *Effects of pindolol, sotalol and the optical isomers of propranolol on muscle action potentials and depolarization-secretion coupling in the rat* Acta physiol. scand. 1978 102. 357–363.

...scle action potentials and miniature end-plate potential frequency were studied in different concentrations of pindolol, *d*- and *l*-propranolol and sotalol using intracellular microelectrode recording from rat ...renic nerve-diaphragm preparations. *d*- and *l*-propranolol at concentrations of 10 to 40 mg/l and pindolol 20 to 100 mg/l slowed down the rate of rise, prolonged rise and half-decay times and reduced the overshoot of the muscle action potentials. Sotalol had similar effects but only at higher concentrations (100 to ...0 mg/l). The drugs had no effect on the increase in the miniature end-plate potential frequency obtained with depolarization by increased extracellular potassium concentration.

Key words: Optical isomers of propranolol, pindolol, sotalol, action potentials, miniature end-plate potentials, skeletal muscle, microelectrode recording

Pindolol, *d*- *l*- and *dl*-propranolol and sotalol have been shown to possess curare-like post synaptic effects at the neuromuscular junction (Lilleheil and Roed 1971 Werman and Wislicki 1971 Larsen and Teräväinen 1977 Larsen 1977). Pindolol and both optical isomers of propranolol also have presynaptic actions. They depress stimulus-induced release of transmitter quanta from the motor nerve endings (Larsen and Teräväinen 1977 Larsen 1977). Since this effect manifested itself rapidly and was reversible, two alternative mechanisms ...ed to explain it. A reduction of the probability of release following a depolarization of the motor nerve endings, and a reduction of the presynaptic depolarization in the form of a diminished amplitude of the nerve action potential are both possible.

The present study was undertaken in order to investigate these alternatives by studying the effects of pindolol, sotalol and the isomers of propranolol on the depolarization-induced increase in transmitter release and on muscle action potentials. The effect on muscle action potentials is regarded as a model of their membrane effect and is presumed to correlate to the presynaptic action potential

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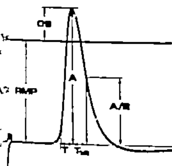


Fig. 1 The variables measured from the action potentials. A = amplitude, OS = overshoot, T = rise time, T = half-decay time. The rate of rise is calculated as A/T. The line in the recording indicates the zero potential, RMP is the resting membrane potential and S is the stimulus artifact

-propranolol was observed to cause qualitatively and quantitatively similar effects as *l*-propranolol on the configuration of the APs, the mean values obtained for the two isomers differing less than 2% from each other at all different concentrations tested. For this reason values of the two isomers are not shown separately in Fig. 3 but represented by their common mean value at each concentration.

Pindolol and sotalol had effects qualitatively similar to those of *d*- and *l*-propranolol on the configuration of the action potentials, but higher concentrations were needed (Fig. 3). The concentrations needed to cause a 20% change from the control value in the variables measured from the action potentials were 4 to 7 times higher for pindolol and 30 to 50 times higher for sotalol than for either *d*- or *l*-propranolol. At the different concentrations tested, the effect of *d*- and *l*-propranolol (10, 20 and 40 mg/l) and pindolol (20, 60 and 100 mg/l) on all the variables measured was statistically highly significant ($p < 0.001$) compared with the control values. Sotalol, at a concentration of 40 mg/l, prolonged the half-decay time and reduced the overshoot significantly ($p < 0.01$). Highly significant ($p < 0.001$) changes in the half-decay time and the overshoot of the action potentials were observed at a concentration of 100 mg/l. Concentrations of sotalol up to 100 mg/l did not have significant effects on the rise time and rate of rise of the action potentials, but the rise time was prolonged and the rate of rise slowed down highly significantly ($p < 0.001$) at 200 mg/l (Fig. 3).

There were no significant differences in the miniature end-plate potential (MEPP) frequency of fibres treated with *d*- or *l*-propranolol or sotalol compared with the control values in solutions containing either 5 mmol/l or 20 mmol/l potassium (Table 1), but the MEPP fre-

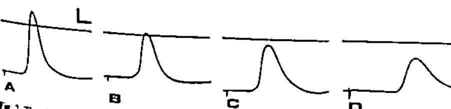


Fig. 2 The effect of different concentrations of *l*-propranolol on the action potentials. A is control. B, C and D are recorded in solutions containing *l*-propranolol 10, 20 and 40 mg/l, respectively. The line indicates the zero potential. Scale: 20 mV and 1 ms. The overshoot of the action potentials is reduced, rise time and half-decay time prolonged and the rate of rise slowed down by *l*-propranolol.

Methods

Altogether 44 Sprague-Dawley rats weighing 190 to 250 g were used. The animals were decapitated under ether anesthesia, and the left hemidiaphragm muscle with the phrenic nerve was removed and prepared further in continuously oxygenated bathing solution. The preparations were then placed in a recording chamber with a fluid capacity of 4 ml, the solution being replaced at a rate of 400 ml per hour. The bathing solution had the following composition (in mmol/l): NaCl 135, $NaHCO_3$ 16.5, Na_2HPO_4 1, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1 and glucose 11. It was equilibrated with a mixture of 95% O_2 and 5% CO_2 and had a pH of 7.35–7.40. A solution containing 20 mmol/l of KCl and 120 mmol/l of NaCl was used to study the increase in miniature end-plate potential frequency in response to depolarization of the nerve endings (Liley 1956, Takeuchi and Takeuchi 1961). The drug to be studied was dissolved in the bathing solution and introduced to the bath from another reservoir. The drugs used were pindolol base (Sandoz Ltd, Switzerland) mixed with tartaric acid 2:1, *d*- and *l*-propranolol hydrochloride (ICI Pharma OY, Finland) and sotalol hydrochloride (Lääke OY, Finland). The preparations were equilibrated in the drug-free solution and in the solutions containing one of the drugs for 30 min before the recording was begun and after the change of solution. All the recordings were made at a bath temperature of 23°C.

Resting membrane potentials (RMP), miniature end-plate potentials (MEPP) and action potentials (AP) were recorded intracellularly with glass capillary microelectrodes filled with 3 M KCl and selected to resistances between 5 and 15 megohms. The potentials were fed through a Mentor N-950 preamplifier and displayed on a Tektronix 5115 storage oscilloscope. The traces were photographed from the oscilloscope screen and measured from magnified negatives. Only those fibres with an initial RMP of at least 65 mV negative inside and which did not depolarize more than 5 mV during the recordings were used. The microelectrode penetration was made under stereomicroscope control about 6 mm away from the end-plate regions when recording APs, and as near as possible to the intramuscular nerves when MEPPs were recorded. The criterion for focal placement of the microelectrode to the end-plate region was the recording of MEPPs with rise time shorter than 1 ms (Nickels 1970).

The MEPP frequencies were calculated first from 10 fibres in a solution containing 5 mmol/l potassium and then from another 10 fibres in a solution containing 20 mmol/l potassium (high K^+ solution) in each preparation. At least 100 MEPPs were counted from each fibre. The MEPP frequency ratio (MFR) was calculated as MFR = (mean MEPP frequency in high K^+ solution) / (mean MEPP frequency in 5 mmol/l K^+ solution) in each of the preparations. Each of the drugs was tested in 4 different preparations and 4 preparations were studied without any drugs.

Muscle action potentials were studied after stimulating the phrenic nerve through a suction electrode with supramaximal current pulses 0.02 ms in duration. The pulses were generated by a Grass S8 stimulator with an SIU 4678 stimulus isolation unit. Only one AP was recorded from each fibre because of frequent fibre damage and microelectrode replacement due to contraction of the fibres. The variables chosen to illustrate the effects of the drugs on the APs are: rise time (T_r), half-decay time ($T_{1/2}$), overshoot and rate of rise of the AP as defined in Fig. 1. Each concentration of each drug was tested in 2 different preparations. Control values were taken before the drug was added.

Student's t-test was used to determine the statistical significance of the differences between the means.

Results

None of the drugs studied caused significant differences in the resting membrane potential recorded in normal bathing solution containing 5 mmol/l potassium. The resting membrane potential recorded without any drug from 85 fibres was 75.8 + 7.2 mV (mean ± S.D.), and that of 70 fibres recorded with pindolol was 74.7 ± 6.9 mV. The corresponding figures were 76.9 ± 7.5 mV for 70 fibres recorded with *d*-propranolol, 74.9 ± 8.0 mV for 70 fibres in *l*-propranolol and 77.1 ± 7.9 mV for 70 fibres in sotalol.

The effect of different concentrations of *l*-propranolol on the muscle action potentials is shown in Fig. 2. *l*-Propranolol caused a dose-dependent reduction of the overshoot of the action potential, as well as a prolongation of rise and half-decay times and a slowing down of the rate of rise (Fig. 2 and 3).

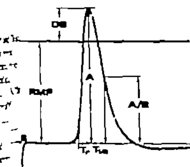


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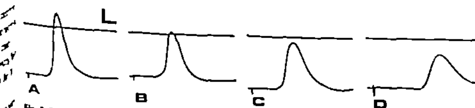


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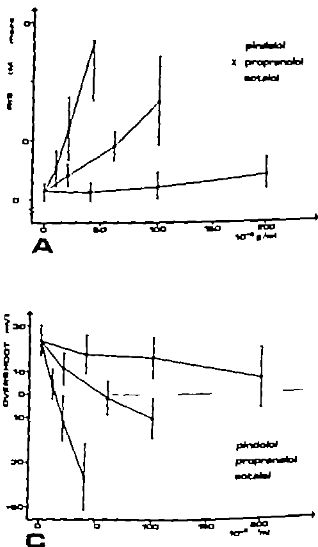
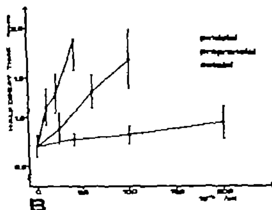


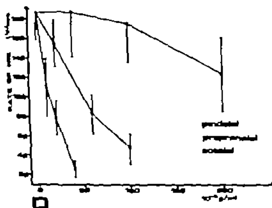
Fig. 3. The effect of pindolol, sotalol and the optical isomers of propranolol on the action potential var is shown in relation to thei concentration. A shows the effect of the drugs on the rise time, B on the decay time, C on the overshoot and D on the rate of rise of the action potentials. Abscissa The conc tion of the drugs. The signs indicate mean and S.D those of control being calculated from 60 fibre those of pindolol and sotalol from 30 f bres each. The v lues of *d*- and *l*-propranolol differed less th from each other at all concentrations tested, nd are therefore represented by their common mean each being calculated from total of 60 f bres, 30 treated w th *d*-propranolol and 30 with *l*-propra

quencies in pindolol containing solutions were higher ($p < 0.01$). The MEPP frequen high K solution (20 mmol/l) increased about 32 to 36-fold (Table I) compared with seen with 5 mmol/l potassium, this increase being of about the same magnitude with al drugs. The depolarization of the muscle fibre membrane, caused by the high extracel potassium concentration, was of the same magnitude in all the expts. (Table 1)

Discussion

All the drugs tested reduced the overshoot, prolonged the rise and half-decay times slowed down the rate of rise of skeletal muscle fibre action potential d *l*-isome





propranolol had the strongest effect on a weight basis, whereas the effect of sotalol was weakest, seen first at concentrations 30 to 50 times higher than those of propranolol. None of the drugs affected the increase in miniature endplate potential frequency in response to depolarization caused by increased extracellular potassium concentration.

The effects of these drugs on the action potentials of skeletal muscle fibres resemble their effects on the cardiac muscle action potentials. In the cardiac muscle fibres 1 to 3 mg/l *dl*-propranolol and 6 to 10 mg/l pindolol reduce the amplitude overshoot, decrease the rate of rise and prolong the rise time (Morales-Aguilerá and Vaughan Williams 1965 Davis and Temte 1968, Singh and Vaughan Williams 1971 Maruyama 1973). These effects are also seen with sotalol, but at higher concentrations, 50 mg/l and over (Singh and Vaughan Williams 1970, Strauss *et al* 1970).

The mechanism by which the drugs affect the muscle fibre action potentials is probably an interference with the ionic conductances of the muscle fibre membrane. Because the drugs were observed to prolong the rise time and to reduce the overshoot of the action potentials a blocking action ...

TABLE I The effect of pindolol, sotalol and the optical isomers of propranolol on the increase in miniature end-plate potential (MEPP) frequency produced by a high concentration of potassium. Each value represents the mean ± S.D. of 4 preparations. MEPP frequency ratios (MFR) have been calculated as (mean MEPP frequency in 20 mmol/l K⁺)/(mean MEPP frequency in 5 mmol/l K⁺) in each of the preparations. RMP is the resting membrane potential.

	MEPP fr. in 5 mmol/l K (s^{-1})	MEPP fr. in 20 mmol/l K (s^{-1})	MFR	RMP in 20 mmol/l K (mV)
Control	1.6 ± 0.29	57.9 ± 5.4	36.2 ± 4.2	56.7 ± 4.3
d-Propranolol (20 mg/l)	1.59 ± 0.30	56.3 ± 5.9	35.5 ± 4.6	54.9 ± 4.5
l-Propranolol (20 mg/l)	1.61 ± 0.27	55.0 ± 5.1	34.7 ± 4.9	55.9 ± 5.3
Pindolol (40 mg/l)	4.8 ± 0.36	81.1 ± 11.[illegible]	32.9 ± 4.2	56.1 ± 4.8
Sotalol (40 mg/l)	1.56 ± 0.31	55.9 ± 7.2	35.8 ± 6.3	55.2 ± 5.0

Significant ($p < 0.01$) difference compared with the control.

1952). The prolongation of the half-decay time may reflect an effect on the potassium channels, but it may also result from a delayed inactivation of sodium channels during excitation (Hodgin and Huxley 1952). In frog atrial muscle *dl*-, *d*- and *l*-propranolol suppress the fast inward sodium current of action potentials, without affecting the potassium current (Tarr *et al.* 1973). In squid axons *dl*-propranolol blocks the impulse conduction by interfering predominantly with the sodium conductance but in addition suppresses also the potassium conductance (Wu 1972, Wu and Narahashi 1973).

Previously it has been shown that both *d*- and *l* propranolol and pindolol, but not sotalol, have presynaptic effects by diminishing the number of acetylcholine quanta released per nerve impulse (Larsen and Teräväinen 1977, Larsen 1977). This presynaptic action of pindolol and the both isomers of propranolol is probably due to the "membrane effect" similar to that in the muscle fibres, i.e. an interference with the ionic conductance of the excitable membranes. On the basis of their effect on the muscle action potentials, it can be expected that pindolol and *d*- and *l*-propranolol also reduce the amplitude of the presynaptic nerve action potentials and thus diminish the presynaptic depolarization caused by a nerve impulse. This leads to a reduced number of transmitter quanta released (Katz and Miledi 1967). A reduction in the probability of release is not likely because the drugs did not decrease the depolarization-induced increase in the miniature end-plate potential frequency.

Sotalol has previously been reported to have postsynaptic but not presynaptic effects (Larsen 1977). On the basis of the present observations, sotalol may however also have presynaptic effects but only at considerably higher concentrations than those needed to produce postsynaptic effects.

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Acta physiol. scand. 1978. 102. 364–367

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Urinary cyclic AMP and vasopressin excretion in rat strains selected for their alcohol intake

By

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Abstract

LINKOLA J and F FYHRQUIST *Urinary cyclic AMP and vasopressin excretion in rat strains selected for their alcohol intake* Acta physiol scand. 1978 *102* 364–367

Urinary excretion of adenosine 3',5'-cyclic monophosphate (cAMP) and immunoreactive arginine vasopressin (AVP) were investigated after water loading and following ethanol loading in two rat strains selected for their voluntary ethanol intake. After ethanol loading ethanol preferring (AA) rats excreted more cAMP but less AVP than water preferring (ANA) rats. The results suggest that the strain difference in cAMP excretion is of renal origin and is not due to vasopressin or parathormone. Differences in the sympathetic nervous activity may be responsible for the difference in cAMP excretion.

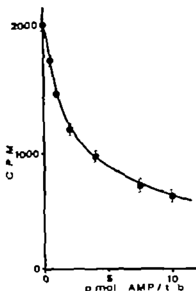
Two rat strains (AA – Alko Alcohol and ANA – Alko Non-Alcohol) have been selected for their voluntary ethanol drinking (Eriksson 1968, Eriksson 1971). Diuretic response to ethanol was lower in alcohol preferring (AA) rats than water preferring (ANA) rats (Linkola *et al* 1977 a). In accord with this, AA rats excreted more vasopressin into urine than ANA rats (Linkola *et al* 1977 a), when ethanol was administered to non-hydrated rats. However, when ethanol was administered to prehydrated rats, the strain difference in urine output was opposite (Linkola 1976). AA rats excreted more urine in that situation.

In this study urinary excretion of AVP was studied during ethanol diuresis in prehydrated AA and ANA rats. Because of the crucial role of cAMP as a cellular mediator of many hormones, among them AVP (Dousa and Valtin 1976), the urinary excretion of cAMP was also measured.

Material and Methods

10 four-month old, male AA and ANA rats of the F_{20} generation, weighing 208–363 g, were used in the experiment. They were fed and loaded with water and ethanol as described earlier (Linkola 1976), except that 20% v/v ethanol was administered 2.4 g/kg b.wt., and urine was collected into plastic tubes in ice bath.

Urine AVP was determined by radioimmunoassay (Fyhrquist *et al.* 1976, Linkola *et al.* 1977a) and urine cAMP by protein binding assay as follows. Fresh bovine adrenals were used as a source of binding protein. They were homogenized in 50 mM Tris/HCl buffer containing 4 mM Na_2EDTA, pH 7.5 (EDTA buffer) as described (Linkola *et al* 1977b). After acetic acid and ammonium sulphate steps described in



1. A standard curve for cAMP. Each point represents of 4 determinations ± S D. CPM = counts per minute.

…ere detail by Akunes and Sövik (1976), protein preparation was dissolved in the EDTA buffer and extensively dialyzed against the same buffer. The dialyzed solution was centrifuged at 15 000 r.p.m. for 30 min. …steps of the preparation of the binding protein were performed below +4°C. After adjustment of the …tein content to 12 mg/ml with the EDTA buffer the binding protein was stored in portions of 1 ml at …°C. For standard cAMP assay 1 ml of the binding protein was diluted with 9 ml of the EDTA buffer …ne samples were diluted 1:50 with the EDTA buffer which was also used as an assay buffer. Other …ents and assay conditions were as described (Linkola *et al.* 1977 b), except 50 µl of diluted urine was …ed. A typical standard curve for cAMP is presented in Fig. 1. For statistical evaluation Student's t-test employed.

Results

Urine output. During water loading ANA rats excreted more urine than AA rats (Table I). When ethanol was administered there was no strain difference in urine output during the first hour but during the following two hours AA rats excreted more urine than ANA rats, but the difference was not statistically significant.

AVP output. Urinary excretion of immunoreactive AVP was more pronounced in AA than ANA rats during the water loading period (Table I). The difference was, however not statistically significant. After ethanol loading ANA rats excreted about three times more AVP than AA rats during the first hour but no significant difference was observed during the following 2 h.

CAMP output. There was no significant difference in urinary excretion of cAMP during water loading period. After ethanol loading AA rats excreted about twice as much cAMP as ANA rats during the first hour (Table I). The same difference continued during the two following hours.

Discussion

Urine output. Higher urine output in ANA rats than AA rats after water loading confirms previous findings (Linkola 1976, Linkola *et al.* 1977). Similarly support is given to the

TABLE I Urine output (ml/kg h), urinary excretion of AVP (pg/kg·h) and cAMP (nmol/kg h) [illegible] water loading (8 11 a.m.) and after ethanol loading (11 12 a.m. and 0–2 p.m) in AA (n [illegible] ANA (n = 10) rats. Means ± S.D p means the significance of the strain difference.

	AA	ANA	
Urine output			
8 11 a.m.	17.0±4 3	2..5±..7	p < 0.005
11 12 a.m.	˜0.9–2.9	˜0.8±5 8	
0– p.m.	7 1±3 1	5..±1.5	
AVP output			
8–11 a m	138 1±114 0	89.3±105 1	
11 1 a.m	95 8±90 0	301 9±184 4	p < 0 01
0– p.m	1..8 9±71 6	117 1±44 4	
cAMP output			
8–11 a.m.	..3.3±3 9	1.3±4..	
11 12 a.m.	19 9±8.5	8.5±4.2	p < 0 005
0–2 p.m	18..±3 0	9 4±3.3	p < 0.001

earlier report (Linkola 1976) that in prehydrated rats ethanol causes stronger diures[illegible] rats than ANA rats. That the strain difference was less obvious than earlier may be [illegible] different prehydration procedure or to differences between rat generations. An earlie[illegible] (Linkola 1976) was made with F rats, while F_{**} rats were used in this work.

AVP output Because urine was collected during one and two hours, possible fluct[illegible] in AVP excretion within short intervals could not be estimated Higher AVP outpu[illegible] rats than ANA rats after water loading agrees with previous results (Linkola 1976, [illegible] *et al* 1977 a). The remarkable increase in AVP output in ANA rats after ethanol [illegible] not seen in AA rats, may partly explain the diuresis of AA and ANA rats (Table I [illegible] kola 1976) because it has been reported that urinary AVP output reflects the plas[illegible] centration of this hormone (Miller and Moses 1971).

The reasons for strong AVP output in ANA rats as compared to AA rats after [illegible] remain unclear AVP release may be suppressed by noradrenaline (Berl *et al.* 1974 [illegible] rats. As suggested (Linkola 1976) it is possible that the primary cause for the diffe[illegible] the diuresis between AA and ANA rats lies not in the release of AVP but, more [illegible] the autonomic nervous system affecting AVP release.

Sodium ions may also contribute to AVP release in ANA rats after ethanol [illegible] because more sodium is apparently accumulated in prehydrated, ethanol loaded A[illegible] than in AA rats (Linkola 1976) Conversely the retention of sodium may also b[illegible] sequence of the actions of AVP in the kidneys (Hornych *et al* 1973).

CAMP output The difference in the urinary excretion of cAMP may be at the le[illegible] to cAMP synthesis or at the level of the factors stimulating cAMP synthesis. Amo[illegible] factors are vasopressin (Dousa and Valtin 1976), parathormone (Chase and Aurba[illegible] and catecholamines (Dousa and Valtin 1976)

Vasopressin is not likely to be responsible for the strain difference in cAMP [illegible] because ANA rats excreted more AVP after ethanol loading than AA rats. Neither [illegible] pressin values alone explain the diuretic response of the rats after water and ethanol [illegible] (Table I). CAMP output indicates that other cAMP mediated processes may also co[illegible] to urine output of the rats.

...ere is no data on parathormone of AA and ANA rats. However unpublished results ...igher plasma calcium concentration and higher urinary calcium excretion of ANA rats ... AA rats suggest that parathormone was not responsible for the strain difference in ...P excretion.

...cause our unpublished observations showed no higher plasma cAMP concentration ...A rats than in ANA rats, it is likely that the strain difference in cAMP excretion is of ... origin. Ethanol stimulates adenylate cyclase in many tissues (Volicer and Gold 1975). ...ever the slight strain difference in cAMP excretion already during water loading indi... that ethanol is not needed to cause difference in cAMP excretion. Catecholamines also ...mulate adenylate cyclase in the kidneys (Dousa and Valtin 1976, Beck *et al.* 1972), resulting ...he release of renin. Our recent unpublished observations indicating higher plasma renin ...ity in AA rats than ANA rats therefore suggest that the actions of catecholamines in ... kidneys may be responsible for the strain difference in cAMP excretion.

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Acta physiol. scand. 1978. 102. 368–373

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Estrogen and the relaxant effect of intramural noradrenaline on calcium induced contractures in depolarized rat uterus

By

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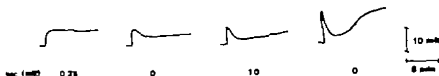
Abstract

BENGTSSON B *Estrogen and the relaxant effect of intramural noradrenaline on calcium induced contractures in depolarized rat uterus* Acta physiol. scand. 1978, *102* 368–373.

The influence of intramural noradrenaline on calcium induced contractures was studied in isolated preparations of rat uterus. The depolarized (1 7 mM KCl) myometrium of oophorectomized rats responded with contraction f llowed by a transient relaxation when exposed to 3 mM calcium. The threshold concentration of calcium, where the transient relaxation began to ppear was between 0.25 nd 0.5 mM. Blockade of the β-adrenoceptors with propra olol o noradrenaline depletion with reserpine completely removed the transient relaxation, indicating that the latter was due to release of intramural noradrenaline. Estrogen treatment abolished the elaxant effect of intramural noradrenaline whereas progesterone was ineffective in this respect. Preparations from rats in natural estrus responded like estrogenized tissues, and diestrus preparations behaved as uteri of oophorectomized rats witho t estrogen treatment.

The isolated rat uterus, depolarized in an isotonic potassium solution, produces graded contractures in response to calcium added to the external medium (Edman and Schild 1962) The present study showed that the calcium contracture under certain conditions displayed a quite complex pattern Thus, when the influence of estrogen was small, the contractile response was found to contain 3 distinct phases an initial rapid rise of tension, a partial relaxation, and a subsequent slow increase of tension. The different phases of the contraction could possibly be explained on the basis of different sources of activator calcium, for instance one with rapid and another with slow transport of calcium into the contractile system. Quite another possibility is that calcium releases a biologically active substance, stored in the uterus, which causes a transient relaxation and thereby interrupts the otherwise smooth tension rise.

The present paper describes experiments designed to further elucidate the mechanism behind the calcium contracture of depolarized rat uterus. Results show that the transient relaxation occurring during the calcium contracture is caused by released intramural noradrenaline. A preliminary report on this investigation was given previously (Bengtsson



[illegible] Contractile responses to different concentrations of calcium in depolarized (127 mM K) uterine from oophorectomized rat. Calcium concentrations as indicated in the figure.

Material and methods

[illegible] 4 oophorectomized and 10 normal virgin rats (190–200 g) of the Wistar strain. 10 of the operated received 0.5 mg polyestradiol phosphate and 4 were given 5 mg oxyprogesterone caproate as an intramuscular injection immediately after operation. The remaining oophorectomized animals received no hormone treatment and served as controls. They were killed on day 7 after operation. 30 h before the expt. intraperitoneal injection of reserpine (5 mg/kg) was given to 5 of the controls and 5 of the estrogenized rats. In rabbits, this treatment depletes the uterus of noradrenaline within 24–36 h (Owman and Sjö- 1967).

Of the 10 rats with intact ovaries, 5 were in early estrus and the rest in diestrus when used in the expts. Stage of the estrus cycle was determined by histological examination of the vaginal smear.

Preparation and mounting. The rats were killed and quickly exsanguinated. The uterus was isolated, [illegible] Krebs solution (see below), and split open by longitudinal sections at the mesometric borders of [illegible]. The uterine horns were divided transversely into pieces about 15 mm long. These were mounted [illegible] in 30 ml jacketed organ baths and stretched to a tension of 10 mN, which gradually reduced to [illegible] tension of 1–2 mN within 10 min. The muscular tension was recorded isometrically by a Grass [illegible] transducer connected to a Grass P7 polygraph ink-writer.

Experimental procedure. The uterine strips were allowed to equilibrate for 30 min in ordinary Krebs solution. During this time, regular spontaneous contractions appeared in all preparations except those of estrogenized animals. In the latter case, only infrequent contractions occurred (*cf.* Ljung *et al.* 1971). [illegible] preparations were contracted by immersion in potassium-Krebs solution (see below) containing 1.5 [illegible] calcium. After 15 min, the preparations were relaxed by immersion in calcium-free potassium-Krebs solution. The muscles were kept in the calcium-free medium for 30 min before a new contracture was induced by injection of calcium chloride into the bathing solution (final conc. 3 mM). In one series of [illegible] contractures were evoked with calcium in different concentrations (0.25–4.0 mM). Between the contractures, the muscle was bathed in calcium-free potassium-Krebs solution for 15 min.

Solutions. The muscle strips were bathed in Krebs solution, composition (mM): NaCl 119, KCl 4.6, $NaHCO_3$ 20, $CaCl_2$ 1.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2, glucose 11, pH 7.4. The *calcium-free potassium-Krebs* had the following composition (mM): KCl 127, $NaHCO_3$ 20, NaH_2PO_4 1.2, $MgCl_2$ 1.2, EGTA 0.01, glucose [illegible], pH 7.4. The solutions were prepared on the day of the expt. Double-distilled water and analytical grade chemicals were used.

The bathing solutions were kept at 37°C and aerated with a mixture of 5% CO_2 and 95% O_2.

Drugs. Atropine (BDH), estradiol-17β (BDH), oxyprogesterone caproate (Proluton-Depot® Schering AG), polyestradiol phosphate (Estradurin® Leo), propranolol (Inderal® ICI-Pharma), and reserpine (Serpasil® Ciba).

Drugs were injected into the bath in volumes of 0.03–0.1 ml, 55 min before the calcium contracture was induced.

Results

Calcium contractures in uteri of oophorectomized rats

Fig. 1 shows a sequence of contractile responses in a depolarized uterine strip subjected to increasing concentrations of calcium. At 0.25 mM calcium, the tension rose smoothly to a level which then was maintained. Calcium at higher concentrations (0.5–4 mM) did not induce contractures which directly reached a sustained maximum, but evoked a phasic



Fig. 2. Contractile responses to 3 mM calcium in depolarized uterine strips from oophorectomized rats (a) control, (b) in the presence of propranolol (2 10^{-4} M), (c) after reserpinization.

contraction followed by relaxation. The active tension, after reaching a minimum, slowly rose to a sustained level. The initial rise and the subsequent decline of tension became steeper as higher concentrations of calcium were used (*cf.* Fig. 1 b and d), and with repetitive stimulation the duration of the relaxation phase became shorter.

The results shown in Fig. 1 suggested that the complex pattern of the calcium contraction was based on a relaxing substance released from stores in the uterus when calcium was added to the medium. Further evidence in support of this idea was provided by the expts. shown in Fig. 2. A recording of a control response to 3 mM calcium demonstrates a typical transient relaxation of a depolarized uterus (Fig. 2 a). Another strip of the same uterus, incubated with propranolol (2 10^{-4} M) in order to block the β-adrenoceptors of the myometrium, responded to calcium with a smoothly developing contracture, without transient relaxation (Fig. 2 b). The calcium contracture in myometrium of reserpinized rat (see Methods) is shown for comparison (Fig. 2 c). Depletion of neuronal noradrenaline and β-adrenoceptor blockade had identical effects, indicating that the transient relaxation of the calcium contracture in the depolarized rat uterus was due to the release of intramural noradrenaline.

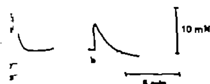
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It might be argued that after estrogen treatment intramural acetylcholine was released in amounts sufficient to compensate for released noradrenaline. However as Fig. 3 d shows the calcium contracture was not changed by blockade of the muscarinic receptors of the myometrium by atropine (10 M). Lack of influence of atropine on the calcium contracture was also noted in non-estrogenized myometrium.

In order to find out whether estrogen exerted an immediate effect on the contractile response to calcium, myometrium from non-estrogenized (and oophorectomized) animals was incubated with 3 / M estradiol-17β for 55 min before calcium was added to the depolarizing solution. However *in-vitro* treatment with estrogen did not prevent the transient decline of tension during the calcium contracture (Fig. 4 a).



Fig. 3. Contractile responses to 3 mM calcium depolarized myometrium from estrogen-treated rats. (a) control, (b) in the presence of 2 10^{-4} M propranolol, ( ) after reserpinization, (d) in the presence of 10^{-4} M atropine.



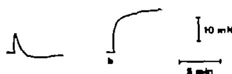


Fig. 4

Fig. 5

4 Contractile responses to 3 mM calcium in depolarized uterine strips from oophorectomized rat. ...ith 3 10^{-9} M estradiol-17β in the bathing solution, (b) injected with 5 mg oxyprogesterone caproate ...ys before the experiment.

5. Contractile responses to 3 mM Ca in depolarized uterine strips from unoperated rats in ( ) diestrus (b) estrus

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Calcium contractures in uteri of rats with intact ovarian function

...e contractile response to calcium in the depolarized rat uterus varied with the animal's ...rus cycle. Thus the uterus of diestrus rats exhibited a transient relaxation of the calcium ...ntracture in very much the same way as uteri of oophorectomized rats (Fig. 5 a), whereas ...myometrium from rats in early estrus responded with a smooth rise in tension i.e. similar ...the response observed in estrogenized preparations (Fig. 5 b).

Discussion

...he rat uterus receives both parasympathetic and sympathetic nerves (Adham and Schenk ...969), and as calcium has the ability to activate the mechanism causing transmitter release ...om nerve endings (for review see Rubin 1970, Kirpekar 1975), a release of both nor...adrenaline and acetylcholine is likely to occur when a preparation of the rat uterus is exposed ...o calcium. Both these substances have been shown to influence the contractility of de...polarized rat myometrium, noradrenaline reduces active tension (Schild 1967), whereas ...cetylcholine induces contraction (Evans *et al.* 1959).

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...Noradrenaline, on the contrary was evidently released in sufficient amounts to counteract ...the contractile effect of calcium in the non-estrogenized uterus, as the calcium contracture ...in this tissue was augmented by both reserpinization and β-adrenoceptor blockade. The ...threshold concentration of calcium where the transient relaxation began to appear indicating ...release of noradrenaline, was between 0.25 and 0.5 mM (Fig. 1). It is of interest to note that ...the release of acetylcholine from cholinergic motor nerves is considerably increased when ...the concentration of extracellular calcium is raised above 0.2 mM (Dodge and Rahamimoff ...1967).

The adrenergic influence on the calcium contracture was removed by treatment with ...estrogen. This is partially explained by two effects of estrogen in the rat uterus: 1 the amount



Fig 2. Contractile respo[nses to 3] mM calcium in depolarized [uterine] strips from oophorectomi[zed rats] (a) control, (b) in the pre[sence of] propranolol (2 10^{-6} M), [(c) after] reserpinization.

contraction followed by relaxation. The active tension, after reaching a minimum, [again] rose to a sustained level The initial rise and the subsequent decline of tension b[ecame] steeper as higher concentrations of calcium were used (*cf* Fig. 1 b and d), and with re[peated] stimulation the duration of the relaxation phase became shorter

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Fig. 3 Contractile responses to 3 mM calcium in depolarized myometrium from estrogen-treated rat[s] ( ) control, (b) in the presence of 2 10^{-6} M propranolol, ( ) after reserpinization, (d) in the presence of [10^{-6}] M atropine.

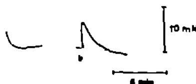




Fig. 4 Fig. 5

4. Contractile responses to 3 mM calcium in depolarized uterine strips from oophorectomized rats, with 3 10^{-4} M estradiol-17β in the bathing solution, (b) injected with 5 mg oxyprogesterone caproate before the experiment.

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Discussion

The rat uterus receives both parasympathetic and sympathetic nerves (Adham and Schenk 1969), and as calcium has the ability to activate the mechanism causing transmitter release from nerve endings (for review see Rubin 1970, Kirpekar 1975), a release of both noradrenaline and acetylcholine is likely to occur when a preparation of the rat uterus is exposed to calcium. Both these substances have been shown to influence the contractility of depolarized rat myometrium, noradrenaline reduces active tension (Schild 1967), whereas acetylcholine induces contraction (Evans *et al.* 1959).

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of noradrenaline is decreased per weight smooth muscle tissue (Oskarsson 1960, Fald 1974) 2. the sensitivity to the relaxant effect of noradrenaline is reduced (Bengtsson 19 However even the combined effect of decreased concentration and reduced sensitn probably insufficient to explain the complete elimination of the influence of intra noradrenaline shown in this study As will be described in a coming paper estrogen al also the release of H noradrenaline in the rat uterus (Bengtsson 1977 b).

Estrogen did not remove the transient relaxation of the calcium contracture when a to the uterus *in vitro* However a number of physiological effects of estrogen require lo conditions to develop and have not been reproduced *in vitro* (for review see Spaziani 1

The myometrium of rats in natural estrus responded to calcium in a way similar to of estrogen treated animals, whereas the diestrus preparations behaved like the un oophorectomized rats without estrogen treatment This is understandable, as the e phase is preceded by the maximum estrogen secretion of the estrus cycle, and as die occurs after 2–3 days of minute estrogen secretion (Yoshinaga *et al* 1969 Brown-G *et al* 1970, Shaikh 1971) The present finding that the relaxant effect of intramural adrenaline is absent during estrus agrees with an earlier report of Deis and Pickford (19 Investigating contractions of the rat uterus *in situ* they found that the contractile act of the diestrus uterus was greater when the sympathetic nerves were blocked, sugges active adrenergic inhibition at this time In contrast, in estrus uterine contractions w unchanged during sympathetic blockade, indicating that inhibitory sympathetic impul were eliminated during this phase

Progesterone, contrary to estrogen had no sympatholytic action in the present ex In fact given together with estrogen, progesterone would probably exert an indirect sy pathomimetic effect, as gestagens, possibly by reducing the number of estrogen recepto have an antiestrogenic effect in the uterus (for review see Brenner and West 1975).

I wish to thank Professor K. A. P Edman for advice and helpful criticism during the course of the i vestigation, Professor K. E. Andersson for helpful discussions and criticism of the manuscript and M Christina Olsson for skilful technical assistance.

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[illegible] heart, simultaneous inhibition of α- and β-receptors was required to abolish the PGE [illegible] caused by NA (Wennmalm 1975).

[illegible] the present study the PGE release following infusion of NA and other sympathomi-[illegible] amines was studied in the isolated rabbit heart in both the absence and the presence [illegible] adrenolytic drugs. The aim was to find out whether sympathomimetic drugs, when releas-[illegible] PGE, act directly on some type of adrenoceptive mechanism in the tissue, or indirectly [illegible] inducing e.g. contraction of the organ.

Methods

Technique of heart perfusion

[illegible] of mixed strains and sexes were used. The weight of the animals varied from 1.4 to 2.5 kg. They [illegible] killed by [illegible] blow on the head and exsanguinated by cutting the left carotid artery. The heart was [illegible] removed and transferred to the perfusion apparatus, [illegible] here it was perfused according to Langen-[illegible] with Tyrode solution of the following composition (in mM): N[illegible] 149, K [illegible] 2.7, Ca [illegible] 1.8, Mg^{2+} 1.0, [illegible], HCO_3 12, PO_4 0.4, glucose 5.5. The solution was continuously aerated with 5 [illegible] CO_2 in O_2 [illegible] pH of 7.4–7.5. The perfusion pressure [illegible] 60 cm H_2O and the temperature 37 C. The apex of the [illegible] was connected to [illegible] strain gauge transducer and heart rate and contractile force were recorded on [illegible] Model 5 D Polygraph. The heart rate recording [illegible] as repeatedly calibrated during the course of the [illegible]. The strain gauge transducer [illegible] linear in the load range of 0–5 pounds, and thus permitted the qualita-[illegible] recording of changes in contractile force during the expts. All expts. were started after the heart had [illegible] spontaneously beating in the perfusion apparatus for 10–15 min. During the experiments the effluent [illegible] the organ was collected in consecutive 10 min periods for 40 min.

The sympathomimetic drug to be infused was administered with an infusion pump connected to [illegible] can-[illegible] close to the aorta. The infusion rate [illegible] chosen to produce [illegible] final concentration of the drug of 10^{-6} M. [illegible] experiments the α-adrenoceptor inhibitor phentolamine or the β-adrenoceptor inhibitor propranolol [illegible] added to the Tyrode solution from the beginning of the experiment, at [illegible] concentration of 5 [illegible] 10^{-6} M. [illegible] infusion was performed during the second 10 min effluent collection period. The first, third and [illegible] effluent collection periods served as controls, and the mechanical activity and the PGE overflow [illegible] well as the coronary flow rate during these periods were regarded as basal.

Analysis of prostaglandins

[illegible] effluent from the heart [illegible] acidified, and its content of lipids was extracted twice [illegible] equal amounts [illegible] ethyl acetate. After washing of the organic phase to neutral reaction, it was evaporated to dryness, [illegible] the residue was dissolved in [illegible] small amount of saline. The PGE-like activity of the dissolved residue [illegible] tested against known amounts of PGE_2 on the rat stomach strip (Vane 1957), mounted in an organ bath [illegible] 4.5 ml volume, containing 37°C Tyrode solution of the composition mentioned above. T[illegible] the solution [illegible] added phentolamine (7 [illegible] 10^{-7} M), propranolol (8 [illegible] 10^{-7} M), atropine (10^{-7} M), methysergide (6 [illegible] 10^{-}[illegible]) and diphenhydramine (7 [illegible] 10^{-7} M), in order to block activity in the strip due to the presence in the [illegible] extract of NA, A, acetylcholine, 5-hydroxytryptamine or histamine.

The sensitivity of the assay organ was accepted when 5 ng of PGE_2 induced [illegible] definite and reproducible [illegible] contraction. As [illegible] consequence an outflow rate of PGE from the heart corresponding to 1 [illegible] g PGE_2/min [illegible] could be accurately measured. Each lipid sample was tested twice, the intra-individual variation usually [illegible] less than 15 [illegible]. The recovery of PGE was regularly checked by the use of external standards of PGE_2, [illegible] was found to range between 55 and 80 [illegible]. The values given for outflow of prostaglandins are expressed [illegible] PGE_2 equivalents and are not corrected for losses during the purification procedure. For identification [illegible] of PGE in the effluents from the hearts two extracts were subjected to thin layer chromatography (Green [illegible] and Samuelsson 1964).

Statistical analysis

Values in the text, tables and figures are presented as mean ± S.E. F[illegible] parenthesis [illegible] brackets indicate number [illegible] of expts. For estimation of the change in prostaglandin outflow caused by drug infusion the ratio between [illegible] the outflow during the infusion period and the mean outflow during the preceding and following period [illegible] was calculated. Student's [illegible] test for paired differences has been used when applicable.

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Prostaglandin mediated inhibition of noradrenaline release
IV Prostaglandin synthesis is stimulated by myocardial adrenoceptors differing from the α and β-type

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Abstract

WENNMALM Å. and T BRUNDIN *Prostaglandin-mediated inhibition of noradrenaline release IV Prostaglandin synthesis is stimulated by myocardial adrenoceptors differing from the α and β-type* Acta physiol scand 1978 *102* 374–381

The release of prostaglandi E elicited by sympathomimetic amines was studied in the isolated rabbit heart. The hearts were prepared according to Langendorff with conventional recording of stroke frequency and contractile force. Assays were made of the outflow of PGE during exposition to equimolar concentrations of methoxamine, noradrenaline, adrenaline and isoprenaline in the absence and in the presence of phentolamine r propranolol. Noradrenaline caused an almost four-fold increase in the basal outflow of PGE from the heart, while methoxamine (an α-adrenoceptor agonist) and isoprenaline (a β-adrenoceptor agonist) were both ineffective in this respect Thus, the PGE-releasing capacity of the drugs as not correlated to their ability to activate α or β-adrenoceptors. Furthermore, no relation as obtained between the PGE release induced by the drugs and the increase i heart rate and contractile force elicited by them. It is suggested that sympathomimetic drugs trigger PGE synthesis and release in the rabbit myocardium following activation of a hitherto unobserved adrenoceptive mechanism, optimally stimulated by NA

Sympathetic nerve stimulation has been shown to cause release of prostaglandin E (PGE) from different tissues and species, e.g the rat epididymal fat pad (Shaw 1966), the dog spleen (Davies *et al* 1967 Gilmore *et al* 1968) and the rabbit heart (Wennmalm and Stjärne 1971) Since noradrenaline (NA) (and adrenaline (A) as well) upon infusion have been shown to cause PGE release (Gilmore *et al* 1968 Junstad and Wennmalm 1973) the factor triggering the release is probably the neurotransmitter liberated

While it has thus been demonstrated in different tissues and species that adrenoceptor agonists may release PGE from sympathetically innervated organs the type of receptor mechanism underlying this release is incompletely known In the dog spleen the PGE release (and the mechanical response) caused by A failed to materialise at all upon pretreatment with phenoxybenzamine (Davies *et al* 1967), indicating that α-adrenoceptor activation and or contraction of the organ might be involved in the release of PGE [illegible] perfused

Table II. Chronotropic and inotropic responses to infusion of sympathomimetic amines at 10^{-6} M in the absence or presence of adrenolytic agents. The chronotropic response is expressed as the maximal percent increase in heart rate during drug infusion compared to the heart rate prior to the infusion. The inotropic response is expressed as the maximal recorded increase in contractile force during the drug infusion compared to the mean contractile force before and after the infusion.

Drug(s)	Positive chronotropic response	Positive inotropic response
[illegible]	0 (5)	0 (5)
Noradrenaline		
no adrenolytic drug present	33 ± 9 (4)	63 ± 14 (4)
+ phentolamine 5 10^{-6} present	37 ± 1 (4)	0 (4)
propranolol 1 10^{-6} present	0 (4)	50 ± 9 (4)
Adrenaline		
no adrenolytic drug present	66 ± 8 (5)	69 ± 9 (5)
+ phentolamine 5 10^{-6} present	59 ± 4 (7)	50 ± 12 (7)
propranolol 1 10^{-6} present	0 (10)	63 ± 8 (10)
Isoprenaline	73 ± 12 (4)	22 ± 8 (6)

Noradrenaline

Infusion of NA induced a marked increase in heart rate and contractile force. Omission of the drug infusion was followed by restitution of the basal heart rate, while the contractile force faded somewhat. During the infusion of NA an almost 4-fold increase ($P < 0.01$) in the outflow of PGE was observed, which also returned towards the basal level after the end of the NA infusion.

In the presence of the α-adrenoceptor blocker phentolamine the frequency response of the heart to NA was unaffected, while the contractile response disappeared completely. The NA induced overflow of PGE was almost abolished in the presence of phentolamine (Fig. 2).

Following β-blockade with propranolol no frequency response to NA was obtained, while the inotropic response remained almost unchanged. The overflow of PGE induced by NA infusion was slightly but insignificantly reduced.

c. Adrenaline

Infusion of adrenaline elicited a pronounced chronotropic and inotropic response. The outflow of PGE was markedly ($P < 0.001$) increased, by 160 ± 40% (Fig. 1).

Phentolamine did not significantly affect the chronotropic or inotropic responses to A. The PGE overflow observed during A infusion was markedly reduced, only an insignificant elevation was noted when A was infused in the presence of phentolamine.

TABLE I Prostaglandin outflow, expressed in ng/min, from the perfused rabbit heart during 4 consecutive 10 min periods. The respective sympathomimetic drugs were infused during the second 10 min period (10–20) at a rate producing a final concentration of 10^{-6} M. Outflow of prostaglandin significantly differing from the mean outflow during the preceding and the following period indicated by: * = P < 0.05 ** = P < 0.01 *** = P < 0.001

Perfusion conditions	I	II	III	IV
	0–10	10–20	20–30	30–40
1 Methoxamine	3.4 ± 1.0 (5)	2.0 ± 0.7* (5)	3.0 ± 0.8 (5)	2.1 ± 0.5 (5)
2 Noradrenaline				
a. no adrenolytic drug present	2.0 ± 0.4 (4)	11.0 ± 2.1 (4)	—	4.8 ± 1.7 (4)
b. phentolamine 5 · 10^{-6} M present	3.3 ± 0.7 (4)	7.0 ± 0.8 (4)	—	3.1 ± 0.8 (4)
c. propranolol	3.4 ± 0.5 (4)	[illegible].0 ± 0.7 (4)	—	2.6 ± 0.6 (4)
3 Adrenaline				
a. no adrenolytic drug present	3.4 ± 1.0 (5)	7.5 ± 0.8 (5)	—	3.9 ± 0.9 (5)
b. phentolamine 5 · 10^{-6} M present	2.4 ± 0.4 (8)	3.8 ± 0.9 (8)	4.0 ± 1.0 (8)	3.8 ± 1.0 (8)
c. propranolol 5 · 10^{-6} present	1.9 ± 0.3 (10)	2.7 ± 0.6 (10)	3.2 ± 0.8 (8)	3.5 ± 0.7 (8)
4 Isoprenaline	1.7 ± 0.4 (6)	2.3 ± 0.8 (6)	3.3 ± 0.4 (6)	3.9 ± 0.3 (6)

d. Drugs

The drugs used were: methoxamine HCl (Burroughs Wellcome & Co), noradrenaline bitartrate and adrenaline bitartrate (Sigma Chemical Co), isoprenaline (Minnesota 3M Labs.), phentolamine (Regitine®, Ciba) and propranolol (Inderal®, ICI).

Results

The spontaneous beating rate of the hearts in the perfusion apparatus ranged between 115 and 170/min. It usually declined somewhat during the course of the expts., as did the contractile force. The coronary flow ranged between 18 and 60 ml/min at the beginning of the expts. and was also found to be moderately decreased towards the end of the perfusion. The initial spontaneous outflow of PGE in the perfusate ranged between 0.8 and 5.1 ng/min. The outflow varied insignificantly between consecutive periods of effluent collection, provided the heart was allowed to beat spontaneously. In most expts. a weak tendency of the outflow of PGE to decline during the course of the expt. was observed. Thin layer chromatography of two lipid extracts revealed that its biological activity chromatographed with PGE_2. The outflow figures for PGE at rest and during infusion in the absence as well as in the presence of adrenolytic drugs, are presented in Table I. The chronotropic and inotropic responses to the amines are presented in Table II.

a. Methoxamine (MA)

Infusion of MA did not change the beating rate or the contractile force of the heart. The overflow of PGE during the infusion was reduced (P < 0.05) by 40 ± 11 % (n = 5), but returned to the pre-infusion level after the drug infusion.

Discussion

...s sympathetic nerves discharge and NA is liberated from the nerve endings, the synthesis ...rdease of prostaglandins seems to be stimulated. This has been demonstrated as an ...sed overflow of PGE in the venous effluent from various tissues in response to sym... ...etic nerve stimulation (Davies *et al.* 1967, Gilmore *et al.* 1968, Shaw 1966, Wennmalm ... Stjärne 1971). The mechanisms underlying this PGE release have not been clarified. ...is not yet known whether the PGE is released from the nerve endings or from the ...vated tissue. A direct release from the nerve endings into the synaptic cleft seems not ... probable since it has been shown that not only sympathetic nerve discharge but also ...sion of NA (and A as well) elicits PG release into the effluent from sympathetically ...rvated organs (Gilmore 1968, Junstad and Wennmalm 1973). Consequently it appears ...e likely that the synthesis and release of PGE during sympathetic nerve stimulation ...rs as one of the receptive functions triggered by the released neurotransmitter substance. ...not known for certain whether or not this receptive function involves the same part of ...cell surface in the synaptic cleft which is activated by the chemical stimulus and is conven...ally named the adrenoceptor. However, it has been reported, that pretreatment of the ...spleen with phenoxybenzamine, an adrenoceptor blocking agent, inhibits the release ...PG induced by the infusion of A (Davies *et al.* 1967). This might indicate that the PGE ...ase should be bound to some of the adrenoceptor functions, possibly that commonly ...rred to as the α-receptor. An alternative explanation would be that the mechanical ...ponse in the form of muscular activity directly or indirectly should facilitate the release ...PGE. If so, the inhibited release of PGE by phenoxybenzamine in the dog spleen experi...nt mentioned above (Davies *et al.* 1967) should be secondary to the lack of contractile ...response to A. Supporting this latter alternative is the finding by Block and Vane ...(1973) that fibrillation in the isolated rabbit heart abolishes its basal release of prostaglandin...substances. These authors concluded that the PGE release in the rabbit heart is cor...ated to the mechanical activity of the organ. On the other hand, hypercalcemia or hypo...emia have been shown to cause a considerable inotropic response in rabbit hearts without ...ecting their basal release of PGE (Wennmalm 1975). These results do not support the ...a of a causal relationship between PGE release and mechanical activity and prompt a ...re detailed analysis of the adrenoceptor role for the NA induced release of PGE in the ...art.

The most efficient agent for the release of PGE tested in the current series was NA which ...infusion increased the overflow of PGE in the effluent to almost 4 times the basal value. After the infusion the PGE overflow returned to the resting level. Infusion of A also sti...ulated the synthesis and release of PGE, the overflow increasing to more than twice the ...basal value. Also after A infusion the increased PGE outflow returned to the initial level. In parallel to facilitating the PGE outflow infusion of NA and A elicited its well known ...stimulation of stroke frequency and contractile force, which persisted during the whole ...period of drug infusion. However the relationship between the overflow of PGE and the ...concomitant increase in the mechanical activity of the heart was not causal, for the following ...reason. After addition of phentolamine, and inhibitor of the α-response in the adrenoceptor

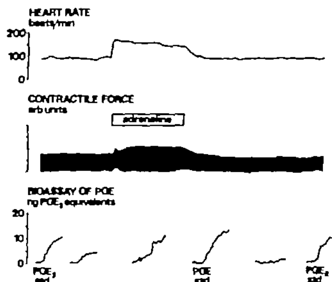


Fig. 1 Perfused rabbit heart The upper two tracings show heart rate and contractile force at rest and during infusion of adrenaline (maintained during the interval indicated by the box). The lower tracing demonstrates the contraction of the rat stomach strip when exposed to ng of PGE$_1$ (PGE$_1$ std), or to a fraction of the dissolved extract of the effluent from the heart, collected during the 10 min period corresponding to the bioassay tracing

The β-blocking agent propranolol completely counteracted the chronotropic response to A, but did not affect the inotropic response to the drug. No increase in the release of PGE was obtained when A was infused in the presence of propranolol

d Isoprenaline (IP)

IP elicited the most marked positive chronotropic response of the sympathomimetic amines investigated The inotropic response to IP was moderate. IP did not affect the outflow of PGE from the heart (Fig. 2)

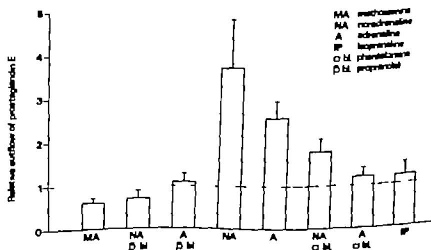


Fig. 2. Outflow of prostaglandin E from the isolated rabbit heart during infusion of sympathomimetic amines in the absence and in the presence of adrenolytic agents. The PGE outflow is expressed as the ratio between the outflow during drug infusion period and the mean outflow during the preceding and the following period. Relative outflow figures under the broken horizontal line consequently indicate that no increase in PGE outflow was obtained during the drug infusion. The different sympathomimetic drugs are arranged from the left t the right in decreasing α-agonistic and incre[...] [...]lytic effect.

echolamines, NA and A. Thus, in this respect the division of the adrenoceptor ... β-types appears unuseful. The view of the adrenoceptor being a single physiologi... s been proposed by others (Ahlquist 1973).

...nary the current results demonstrate that adrenaline and noradrenaline elicit ... PGE from the rabbit myocardium. This effect by the two drugs is clearly distin... from the positive chronotropic and inotropic effects caused by them. It is therefore ... that the rabbit myocardium contains a hitherto unknown adrenoceptive mech... ...ionally stimulated by noradrenaline which on activation facilitates the bioforma... ...E in the tissue.

...was supported by the Swedish Medical Research Council, project 04X-4341 and by Magnus ...ndsx

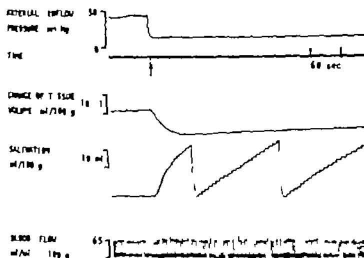
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to the perfusate prior to the infusions of NA or A, the increase in PGE outflow elicited by the amines was markedly reduced, although the chronotropic response to either drug and the inotropic response to A was unaffected Furthermore, IP which on infusion elicited pronounced increase in heart rate and contractile force, did not affect the overflow of PGE from the heart. Thus, the results do not support the hypothesis that the PGE outflow in the rabbit heart is due to the mechanical activity of the organ. On the other hand, it is demonstrated that the PGE releasing effect of NA and A is decreased by the pharmacological agent which blocks the α response of the adrenoceptor which might support that at least part of the receptor function could be involved in the initiating mechanism for PGE release.

It thus appears that the rabbit myocardium contains receptors which can be activated by NA or A to stimulate the formation and release of PGE. Such receptors are by definition adrenoceptors. The current expts. permit a comparison between such PG-adrenoceptors and the conventional adrenergic receptors for electro-mechanical or metabolic responses. The latter are denoted, according to the classification by Ahlquist (1948), as α or β, depending on their relative responsiveness to different sympathomimetic amines most closely related to A which at the time when this classification was suggested, was still believed to be the sympathetic neurotransmitter substance. Concerning the myocardial adrenoceptors, it was originally proposed by Ahlquist that they are of the β-type (Ahlquist 1948). Later strong evidence has been presented supporting the existence of myocardial α-receptors as well (Govier 1968, Rabinowitz *et al* 1975) The physiological significance of α-receptors in the heart has, however been disputed (Nickerson 1973). In the present study the α-adrenolytic agent phentolamine completely inhibited the inotropic response to NA, and the β-adrenolytic agent propranolol completely inhibited the chronotropic but not inotropic responses to NA and A. These results support the hypothesis that the mechanical responses of the heart to catecholamines are not elicited via activation of myocardial β-adrenoceptors only Since the scope of the current investigation was to study the adrenoceptive mechanism behind myocardial PGE synthesis stimulation these observations on the adrenergic mechanical responses of the heart, although *per se* interesting, were not studied more in detail.

MA a drug considered to act only on α-adrenoceptors, was found inefficient to induce chronotropic or inotropic response in the present study The effect of MA infusion on the overflow of PGE in the myocardial effluent was slightly negative. This shows that a selective α-agonist is not the adequate stimulus for PGE release, in spite of the fact that this release was readily inhibited by the α-blocking drug phenoxybenzamine (Davies *et al.* 1967). The α-receptor blocking agent thus seemed to be able to inhibit other functions than the activation of α-receptors. A similar inhibition of the NA or A-induced PGE release was found after pretreatment with propranolol, known as a potent inhibitor of the β-adrenergic response. The selective stimulation of β-receptor activity by IP did however not stimulate any PGE release. Obviously both kinds of pharmacological adrenoceptor inhibitors were demonstrated to block the PGE release although none of the artificial α- or β-agonistic stimulant drug were able to mimic the PGE releasing action of biological monoamines. In consequence the receptor for PGE release could not be identical with that conventionally named α or β-receptor Instead the results indicate that concerning the release of PGE the adrenoceptor consitutes a single functional unit that is by the physio



1.1. Original tracings illustrating typical decrease of tissue volume occurring concomitant to the initial, rapid phase of salivation evoked by parasympathetic stimulation (20 Hz; arrow) of the cat submandibular gland perfused at constant blood flow. pen recording salivation reset to control repetitively.

during the initial 25–30 s and then gradually diminished to be completed within 60 s, after this phase, tissue volume instead showed a slight, continuous increase.

There was thus a close relation in time between the early rapid phase of salivation and the observed decrease of tissue volume in the depicted experiment. The cumulative decrease of tissue volume (Fig. 1) was 12.5 ml/100 g tissue. During the same period (~60 s) a total of 24 ml/100 g tissue of saliva was expelled. It seems reasonable to assume that the observed decrease of tissue volume reflects a corresponding output of saliva, an assumption supported by the finding that there was no significant change of tissue volume (regional blood content) in response to papaverine induced maximal dilatation of the constant flow perfused vascular bed. The remaining amount of saliva produced during the first minute of stimulation must have been derived from the blood and will then amount to 11.5 ml/100 g tissue (24 minus 12.5 ml/100 g). This figure corresponds almost exactly to the later steady state rate of secretion, which was 11.6 ml/min 100 g. A similar close correspondence was found also in the other stimulation experiments and it seems unlikely to be fortuitous but may indicate that the rate of fluid mobilization from plasma for subsequent saliva formation was approximately constant from the commencement of stimulation.

In summary it appears that the initial, rapid outflow of saliva in response to parasympathetic activation is partly due to mobilization of fluid from the gland as revealed in terms of a decline of tissue volume, and partly due to formation of saliva from fluid mobilized from the blood. Circumstantial evidence suggested that the latter process occurred at an approximately constant rate during stimulation. The decrease of tissue volume may to a significant extent reflect expulsion of preformed saliva from the salivary ducts caused by contraction of the myoepithelial cells (Emmelin *et al.* 1974, 1976). It may also reflect osmotic attraction of cellular and/or interstitial fluid to the saliva (Schneyer 1975).

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From the Department of Physiology and Biophysics, University of Lund, Sweden

Dynamics of saliva secretion and tissue volume changes during parasympathetic stimulation in the constant flow perfused cat submandibular gland

By

JAN LUNDVALL and JAN HOLMBERG

The secretion evoked by parasympathetic stimulation of salivary glands is rapid initially a then slows down In a recent study (Lundvall and Holmberg 1977) the initial rapid rate salivation from the cat submandibular gland was found to coincide in time with a decre of the tissue volume of the gland The decline of tissue volume was present despite th regional blood volume in the gland must have increased during the evoked vasodilat response In the present investigation an attempt was made to study in some detail t quantitative relation between tissue volume changes and rate of salivation in response parasympathetic stimulation The experiments were performed under conditions of consta blood flow perfusion in order to minimize alterations of the regional blood content in t gland.

The submandibular gland was dissected free unilaterally i 4 cats anesthetized with chloralose-urethane T salivary duct was cannulated w th a polyethylene catheter Blood was shunted from the carotid artery m Harvard peristaltic pump t the gland lar artery Venous outflow from the gland as diverted about an optical drop-recorder un t for continuous registration of blood flow and was then returned to the som via a funnel connected to the right femoral vein. The distal end of the severed chorda-lingual nerve f tted in bipola ring electrode. The gland was placed i hermetically sealed, temperature controll (38°C) plethysmograph f lled with Tyrode solution. Arterial nflow pressure was measured from a T-tub in the arterial shunt The nerve was stimulated with square w ve pulses of ms duration at a rate of 20 H The drops of saliva fell into a cup, the weight of which was continuously registered with a transducer Th weight changes were transformed i to volume changes by calibration. Papa erine was infused close arterial in dose causing maximal vasodilatation in order to study the effect on tissue olume of vasodilatation such. Arterial blood pressure, changes of tissue ol me rate of salivation and blood flow were registered o a Grass Polygraph.

Fig. 1 shows an original record from a typical experiment Blood flow was held constant a 65 ml/min 100 g glandular tissue Control blood pressure was 135 mmHg and it fell ab ruptly upon chorda activation (20 Hz arrow) to a maintained low level of about 43 mmHg. It can be seen that tissue volume started to decrease almost immediately upon commence ment of stimulation and that, with a few seconds delay salivation was induced. The saliva production was rapid initially and then gradually declined to attain a maintained, approx

Acta physiol. scand. 1978, 102, 385–393

From the Institute of Physiology, University of Lund, Sweden

Polyamines and nucleic acids in the mouse kidney induced to growth by testosterone propionate

By

Sig Henningsson, Lo Persson and Elsa Rosengren

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Abstract

Henningsson, S., L. Persson and E. Rosengren. *Polyamines and nucleic acids in the mouse kidney induced to growth by testosterone propionate.* Acta physiol. scand. 1978, *102*, 385–393.

Daily injections of testosterone propionate to castrated mice resulted in a striking increase in kidney weight. Renal putrescine rose sharply and the amounts of spermidine and spermine were also increased. The activity of ornithine decarboxylase was enhanced to values of more than 1 000 times the control level within a few days of testosterone substitution. A moderate and temporary increase in the activity of the putrescine-activated S-adenosyl-L-methionine decarboxylase was observed. Testosterone injections produced large increases of renal RNA but only minor changes in DNA. It is apparent that in mice distinct alterations in polyamine metabolism occur during the development of renal hypertrophy induced by testosterone administration.

Key words: Polyamines, nucleic acids, testosterone propionate, renal hypertrophy

A distinct difference in the rate of formation and excretion of putrescine between male and female mice has been disclosed (Henningsson and Rosengren 1975, 1977). This difference was accounted for by a high activity of ornithine decarboxylase, the enzyme catalyzing the formation of putrescine, in the male kidney. Castration produced a rapid decrease in ornithine decarboxylase activity, whereas on the other hand substitution with testosterone resulted in an increased formation of putrescine.

The significance of changes in the levels of putrescine and the polyamines, spermidine and spermine, and the activities of their synthesizing enzymes has recently been much discussed. The biosynthesis and the accumulation of putrescine and polyamines undergo striking changes in processes related to rapid cell growth, e.g. in liver at regeneration after partial hepatectomy or growth hormone administration, in developing chick embryo, in renal and myocardial hypertrophy and in malignant growth (for ref. see Raina and Jänne 1975, Tabor and Tabor 1976). These and related observations have called attention to the possibility of polyamines being implicated in RNA and DNA metabolism. However, the physiological role of these amines in mammalian tissues is unknown.

Like workers before us, we observed that in mice castration resulted in a considerable

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[illegible] assay of putrescine, spermidine and spermine in kidney extracts

[illegible] separation and quantitative estimation of the amines in kidney extracts were carried by high resolution liquid chromatography using the automatic amino acid analyzer LKB-BIOCAL. The method as described by Hemmingson (1977), was used [illegible] ith minor modifications.

[illegible] and determination of nucleic acids and protein

[illegible] content of RNA and DNA was performed by the Schmidt and Thannhauser (1945) procedure as [illegible] by Schneider (1946). The kidney tissue [illegible] as homogenized in 7 volumes of ice cold water. An [illegible] of the homogenate (0.8 ml) [illegible] as precipitated with 2.5 ml of ice cold 10 [illegible] (w/v) trichloroacetic acid. [illegible] was centrifuged at 900 *g* for 15 min at 0°C. The supernatant was removed and the pellet was [illegible] three times with 2.5 ml of 10% trichloroacetic acid. Thereafter the pellet was suspended in 5 ml [illegible] M KOH and the mixture was hydrolysed for 18 h at 37 C. The hydrolysate was neutralized and [illegible] for 30 min at 0°C. Five ml of 5% trichloroacetic acid was added and the mixture was centrifuged. [illegible] was resuspended in another 5 ml of trichloroacetic acid and centrifuged. The combined [illegible] contained the ribose nucleic acid (RNA) fraction [illegible] hile DNA and protein were spun [illegible] the pellet.

[illegible] amount of RNA was estimated using the orcinol method (Mejbaum 1939). Briefly this was achieved [illegible] the RNA extract with orcinol after [illegible] hich the mixture was boiled in [illegible] ater-bath for 20 min. [illegible] density was measured in [illegible] Zeiss spectrophotometer.

[illegible] DNA the pellet was suspended in 5 ml of 5 [illegible] trichloroacetic acid and the mixture was [illegible] to 90°C for 15 min. After centrifugation, the pellet [illegible] as resuspended once in trichloroacetic acid. [illegible] supernatants which formed the DNA fraction were mixed [illegible] ith 5.2 M $HClO_4$ and diphenylamine [illegible] reagent (Richards 1974). The resulting solution was incubated [illegible] 30°C for 18 h and DNA [illegible] colorimetrically (Burton 1956).

[illegible] was determined by the Folin procedure (Lowry *et al.* 1951). — The standards used were RNA [illegible] yeast, DNA from calf thymus and for protein determination bovine serum albumin. These [illegible] were purchased from Sigma Chemical Co.

Results

[illegible] in weight and protein mass of the kidney in mice during [illegible] administration

The kidney of the male mouse lost about 30% in weight within 3 weeks of castration, *i.e.* [illegible] weight loss from 387±8.1 mg in 16 normal mice to 275±5.0 mg in 32 castrated ones. [illegible] exposure of castrated mice to testosterone propionate resulted in a marked increase of the kidney weight (Fig. 1). The weight was significantly elevated already 12 h after the first injection. Daily injections of testosterone produced a successive increase in weight during the whole period of observations, this increase was at first steep then more slow. Fig. 1 also shows that simultaneously with the increase of kidney weight a parallel increase in the content of renal protein occurred indicating that the weight increase was due to real growth of the kidney. The total amount of renal protein was reduced from 77.4±2.19 mg in normal mice to 53.9±2.66 mg in castrated ones. It should be mentioned that a small and possibly [illegible] increase of body weight was noted after testosterone administration although not statistically significant.

The effect of testosterone on the levels of RNA and DNA in the kidney

The changes in RNA and DNA contents in the kidney of castrated mice after substitution with testosterone propionate are shown in Fig. 2. After testosterone administration the content of RNA rose steeply during the first 3 days and continued to increase throughout

loss in kidney weight a change which could be reversed by testosterone substitution Sely (1939) was first to describe a renal hypertrophy during testosterone injections. Under andro gen stimulation, the kidney cells become larger without apparent mitotic activity (Pfeiffer *et al* 1940) Kochakian and Harrison (1962) showed that testosterone injections in castrated mice produced a large increase in renal RNA but only minor changes in DNA. Hence, the mouse kidney appears to be an organ of choice in studies on the role of di- and polyamines as related to the metabolism of RNA and protein as discerned from the metabolism of DNA

Methods

Male mice of the NMRI strain were used. They were fed a standard pellet diet and had water ad libitum. Gonadectomy was performed at the ge of 8 weeks. Three weeks later testosterone administration began in the form of daily subcutaneous injections of 200 μg testosterone propionate (British Drug Houses Ltd Poole, England) suspended in 50 μl of rachis oil. Castrated controls were given arachis oil only. Mice subjected to testosterone injections as well as controls were sacrificed at varying times throughout the expts.

Preparation of samples for analysis

Mice were stunned and exsanguinated The removed kidneys, from which the capsules ere gently teased off were blotted on gauze, weighed and promptly placed in a beaker cooled on ice.

Extracts f r assay of ornithine decarboxylase and S-adenosyl-L methionine decarboxylase activities were prepared in the following way The kidneys were minced with scissors and part of the tissue as immediately cautiously homogenized i a Dounce type homogenizer (25 strokes with the pestle) in 7 volumes of cold sodium phosphate buffer (0.1 M pH 7.2) containing EDTA (final conc. 10^{-4} M), dithiothreitol (final conc. 5 10^{-4} M) and glucose (0 2% w/v). The homogenate was centrifuged at 20 000 *g* for 20 min at 4°C. The supernatant was decanted nd used.

Extracts for determination of putrescine, spermidine and spermine in the kidney tissue ere prepared as follows. The minced tissue was homogenized in 9 volumes of solution of 4 ,. sulfosalicylic acid and 0.04 ,. EDTA. The extract was boiled on a water-bath for 30 min whereupon the mi ture as chilled and centrifuged The pH of the sample was djusted to 0–2.5 with NaOH and the extract was filtered through a filter with a pore size of 0.22 μm (Millipore Corp Bedford, Mass. 01730).

Determination of ornithine decarboxylase activity

Ornithine decarboxylase activity was determined measuring the release of $^{14}CO_2$ from carboxyl-labelled ornithine. An aliquot of the enzyme extract corresponding to 5 mg of tissue was incubated in a reaction mixture containing ^{14}C-carboxyl-labelled DL-ornithine monohydrochloride (final conc. 10^{-4} M sp act. 0.5 mCi/mmol), pyridoxal 5-phosphat (final conc. 10^{-4} M) and the same phosphat buffer as used for homogenization. The total volume of the incubate was 1.0 ml. The mixture was incubated for 30 min at 37°C. The reaction was terminated by tipping 0.5 ml of 2 M perchloric acid from a side arm of the reaction vessel. Blanks were treated in the same way except that tissue was excluded. The expelled $^{14}CO_2$ was trapped on a 10 25 mm piece f No 005 Munktell f lter paper prepared with 100 μl of hydroxide of Hyamine 10-X (1 M solution in methanol). Maximal absorption of $^{14}CO_2$ was achieved by continued shaking for another 45 min The filter paper was then placed in counting vessel containing 8 ml of Bray liquid scintillation mixture (Bray 1960) and its radioactivity was measured in Packard Tri-Carb liquid scintillation spectrometer

Determination of putrescine-act ated S-adenosyl-L-methionine decarboxylase activity

Enzyme activity was determined by measuring the release of $^{14}CO_2$ from carboxyl-labelled S-adenosyl-methionine in the presence of putrescine according t the method described by Pegg and Williams-Ashman (1969) The incubation mixture contained enzyme extract corresponding to 75 mg of tissue, putrescine (final conc. 2.5 10^{-4} M), ^{14}C-carboxyl-labelled S-adenosyl-L-methionine (final conc. 10^{-4} M, sp. act. 0.5 mCi/mmol) and the same buffer as used for homogenization. The total volume of the incubation mixture was 1.0 ml and the mixture was incubated for 1 h t 37°C. Blanks were obtained by incubating the same reaction mixture without tissue. The $^{14}CO_2$ evolved was trapped and counted as described above f ornithine decarboxylase.

Table 1. Effect of testosterone administration on the concentrations of RNA, DNA, putrescine, spermidine and spermine in the kidney of castrated mice. The concentrations of RNA and DNA are given mg/g and the concentrations of the amines in nmol/g. Mean and the S.E. of mean are given. Number of observations in parenthesis. a: $p<0.05$ b: p 0.01 c: p 0.001 versus controls.

[illegible]	RNA	DNA	Putrescine	Spermidine	Spermine
[illegible]	5.8±0.09 (16)	5.8±0.14 (16)	21± 2.1 (12)	380±18.0 (12)	855±30.8 (12)
	6.4±0.28 (8)[c]	5.5±0.23 (8)	90± 7.7 (12)[c]	411±22.0 (12)	815± 8.7 (12)
	6.4±0.12 (8)[b]	5.5±0.29 (8)	112± 6.3 (12)[c]	377±19.3 (12)	815±26.7 (12)
	6.9±0.19 (8)[c]	5.0±0.18 (8)[b]	151± 7.0 (12)[c]	401±18.6 (12)	868±17.0 (12)
	7.0±0.17 (9)[c]	5.0±0.11 (9)[c]	159±12.8 (12)[c]	499±29.4 (12)[c]	834±20.4 (12)
	7.2±0.24 (8)[c]	4.7±0.22 (8)[c]	154± 9.9 (12)[c]	460±18.3 (12)[b]	903±20.2 (12)
	7.5±0.13 (8)[c]	4.4±0.06 (8)[c]	139± 7.1 (12)[c]	469±17.7 (12)[c]	1 005±41.0 (12)[b]
	6.9±0.13 (8)[c]	3.9±0.10 (8)[c]	163±15.4 (12)[c]	447±15.2 (12)[b]	935±11.0 (12)[a]
	7.1±0.09 (8)[c]	3.8±0.18 (8)[c]	132± 6.4 (12)[c]	399±10.7 (12)	886±17.5 (12)
[illegible]	6.2±0.24 (5)	4.8±0.15 (5)[b]	49± 8.3 (6)[c]	377±28.1 (6)	874±30.0 (6)

...on of DNA concentration. These changes in the levels of nucleic acids testify to hypertrophy as the major cellular process of the renal enlargement. — After castration the total amount of renal DNA was reduced (Fig. 2 and Table I). However this reduction was proportionally less than the weight loss of the kidney. Consequently the concentration of DNA was increased in castrated mice in comparison with normal mice. The total amount of RNA was reduced after castration while no significant change in RNA concentration was observed.

Ornithine decarboxylase and S-adenosyl-L-methionine decarboxylase activities in castrated mice after testosterone substitution

The rate of putrescine formation, i.e. the ornithine decarboxylase activity in the kidney of normal male mice was 3.5±0.52 nmol/mg protein h (5 determinations). 3 weeks after castration, putrescine formation had fallen and was too low to be detectable (10 determinations). To examine the time course of changes in renal ornithine decarboxylase activity after injections of testosterone propionate, groups of 5 mice were killed at different intervals between 3 h and 21 days after the first injection. Results are summarized in Fig. 3. An elevation in enzyme activity was noted 6 h after testosterone substitution the observed figure was 0.2±0.10 nmol/mg protein h (5 determinations). During the first 3 days the activity rose to well above 200 nmol/mg protein h. Further increase in ornithine decarboxylase activity during the remainder of the experimental period was slight.

S-Adenosylmethionine decarboxylase activity in extracts of kidney from castrated mice was significantly higher than in extracts of kidney from normal mice (Fig. 4). Substitution with testosterone propionate after castration resulted in a transient increase of enzyme activity reaching a maximum of about 30% between days 1 and 3 of treatment. After 7 days the enzyme activity had declined to values below the controls however the difference was not statistically significant.

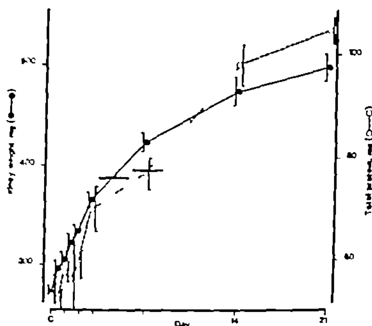


Fig. 1 Weight and total protei the kidney of castrated mice a daily injections of 200 μg testo rone propionate. Each point kidney weight is the mean of observations or more; each p for total protein is the mean values or more. The vertical b define the S.E. of the mean horizontal bars indicate the va for the normal male mice. stands for castrated controls.

the whole period of observations. In contrast with the rapid and sharp elevation of R content, no alteration of DNA content was observed during the first 3 days of treatm Later i.e between 1 and 3 weeks of injections, a moderate enhancement of DNA con appeared.

The concentration of renal RNA gradually increased during the first week of testoster injections and then decreased (Table I). Administration of testosterone gave rise to a red

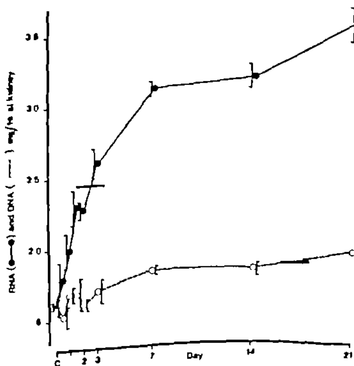
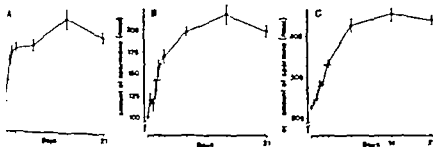


Fig. 2. Total amounts of RNA DNA i the kidney of castr mice after daily injections of μg testosterone propionate. point is the mean ± S.E. of mean of at least eight obse tions. The horizontal bars ind the total amount of RNA DNA in the kidney of normal (5 observations). C stands castrated controls. RNA was nificantly increased at 24 h 0.001) and DNA on the 7 ($p < 0.05$).



...otal amounts of putrescine (A), spermidine (B) and spermine (C) in the kidney of castrated mice ...y injections of 200 μg testosterone propionate. Each point is the mean and S.E. of the mean of 1... ... The horizontal bars indicate values for normal male mice (6 observations). C stands for ... controls.

...jected with testosterone priplonate. After 48 h the total amount of putrescine in the ...was 10 times greater and the concentration was 8 times greater than in the control ... During the following 3 weeks of observation no further alteration in the concentra-... putrescine occurred. However as the kidney weight steadily increased, the total ...t of putrescine was increased. Furthermore, administration of testosterone led to a ...ant increase in the total amounts of spermidine within 12 h and of spermine with-... Within 72 h the total amount of spermidine increased by 70% and that of spermine ...ut 50 %. A further moderate rise of the contents of both amines occurred during the ...ing period of observation. As to the concentrations of spermidine and spermine there ...slight elevation with the highest values on day 7

Discussion

...crease in kidney weight of castrated mice after testosterone substitution was found ...lled an increase in the content of protein in the kidney in good agreement with the ... of Kochakian and Stettner (1948) indicating growth and not edema as the origin of ...enlargement. Selye (1939) and Pfeiffer *et al.* (1940) attribute the enlargement of the ...n after androgen treatment to a hypertrophy of the cells without any apparent mitotic ... This is in accordance with the accumulation of RNA and the limited change in ...content after testosterone administration. These findings agree with the results of ...nar *et al.* (1962) and Kochakian and Harrison (1962).

...dose-response curve (to be published) for testosterone propionate in the range of ...000 μg/mouse day showed a progressive increase in kidney weight and ornithine ...boxylase activity with increasing doses of testosterone. Up to a daily dose of 200 μg ...tosterone propionate the putrescine formation and kidney weight rose rapidly. With ... daily doses the increase in putrescine formation and kidney weight was small. Con-...ntly the dose of 200 μg/mouse day was chosen in the present study

...nistration of the kidneys by testosterone resulted in marked increase of ornithine ...boxylase activity. Decarboxylation of ornithine is considered to be the rate-limiting ...n the biosynthesis of polyamines (Jänne and Raina 1968, Williams-Ashman *et al.* 1969).

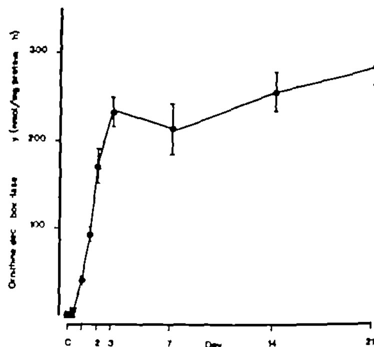


Fig. 3 Ornithine decarb activity (nmol/mg protein the kidney of castrated mic daily injections of 200 µg te rone propionate. Each point mean and S.E. of the me observations except for that control group (C) hich mean of 10 observations.

Contents of putrescine, spermidine and spermine in the kidney of castrated mice after testosterone administration

The total amounts of putrescine, spermidine and spermine as well as the putrescine centration in the kidney of castrated mice were considerably lower than in the kidn normal mice, while concentrations of spermidine and spermine remained unchanged (and Table I)

An early and marked accumulation of renal putrescine occurred when castrated

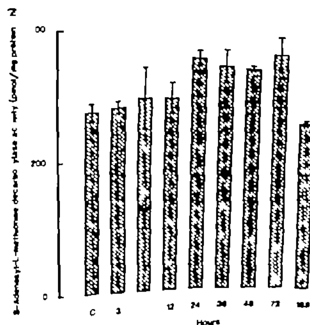


Fig. 4 S-Adenosyl-L-meth decarboxylase activity (pm protein h) in the kidney c trated mice at different time the first injection of 200 µg terone propionate. Each col the mean and S.E. of the m t least 5 observations. C for castrated controls and normal male mice. * p ** p < 0.01 versus controls

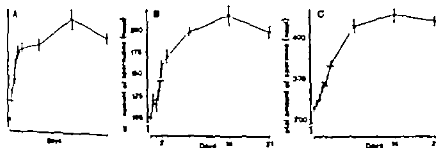


Fig. 3. Total amounts of putrescine (A), spermidine (B) and spermine (C) in the kidney of castrated mice after daily injections of 200 µg testosterone propionate. Each point is the mean and S.E. of the mean of 1 observations. The horizontal bars indicate values for normal male mice (6 observations). C stands for castrated controls.

were injected with testosterone propionate. After 48 h the total amount of putrescine in the kidney was 10 times greater and the concentration was 8 times greater than in the control kidney. During the following 3 weeks of observation no further alteration in the concentration of putrescine occurred. However, as the kidney weight steadily increased, the total amount of putrescine was increased. Furthermore, administration of testosterone led to a significant increase in the total amounts of spermidine within 12 h and of spermine within 36 h. Within 72 h the total amount of spermidine increased by 70 and that of spermine by about 90%. A further moderate rise of the contents of both amines occurred during the following period of observation. As to the concentrations of spermidine and spermine there was a slight elevation with the highest values on day 7

Discussion

The increase in kidney weight of castrated mice after testosterone substitution was found to parallel an increase in the content of protein in the kidney. In good agreement with the results of Kochakian and Stettner (1948) indicating growth and not edema as the origin of renal enlargement. Selye (1939) and Pfeiffer *et al.* (1940) attribute the enlargement of the kidneys after androgen treatment to a hypertrophy of the cells without any apparent mitotic activity. This is in accordance with the accumulation of RNA and the limited change in DNA content after testosterone administration. These findings agree with the results of Kassenaar *et al.* (1962) and Kochakian and Harrison (1962).

A dose-response curve (to be published) for testosterone propionate in the range of 0.1-1000 µg/mouse day showed a progressive increase in kidney weight and ornithine decarboxylase activity with increasing doses of testosterone. Up to a daily dose of 200 µg of testosterone propionate the putrescine formation and kidney weight rose rapidly. With larger daily doses the increase in putrescine formation and kidney weight was small. Consequently the dose of 200 µg/mouse day was chosen in the present study

Stimulation of the kidneys by testosterone resulted in a marked increase of ornithine decarboxylase activity. Decarboxylation of ornithine is considered to be the rate-limiting step in the biosynthesis of polyamines (Jänne and Raina 1968, Williams-Ashman *et al.* 1969).

It is noteworthy that an increase in ornithine decarboxylase activity was detected as earl as 6 h after the injection of testosterone. Further the greatest enhancement of this enzym occurred during the first two days of observation while the changes of the other parameter studied occurred less abruptly This is interesting because a connection between polyamine and growth is assumed to exist (for ref see Russell 1973 Raina and Jänne 1975).

The enhancement of the activity of ornithine decarboxylase was impressive within few days of testosterone treatment the activity rose to values of more than 1 000 times th control level The ornithine decarboxylase activity of the testosterone-stimulated mou kidney appears to be singularly high, as it was found about 5 times larger than the highe value recorded in rat tissues, *i.e* the ovary stimulated by chorionic gonadotropin (Gut and Jänne 1977).

The testosterone induced increase of ornithine decarboxylase activity was accompan by a rise in putrescine content However the increase of putrescine content was out of pr portion to the great enhancement of enzyme activity This could be due to an increa turnover of the amine. Urinary excretion of putrescine was found elevated after testostero administration (Grahn *et al* 1973) Another possibility is that the supply of ornithine inadequate to the enormous activity of ornithine decarboxylase in testosterone-trea mice Interestingly Kochakian (1945) reported an increase in arginase activity (catalyzi the synthesis of ornithine and urea) in androgen stimulated mouse kidney This would res in more ornithine available for decarboxylation and hence for biosynthesis of polyamine

The change in the activity of the second enzyme in polyamine biosynthesis, S-adenos methionine decarboxylase, after testosterone administration was comparatively small. Ho ever the elevated concentration of putrescine, which *in vitro* is known to stimulate activity of S-adenosylmethionine decarboxylase (Pegg and Williams-Ashman 1968), sho give rise to an increased enzyme activity *in vivo* and hence, an elevated synthesis of po amines. It should be noticed that S-adenosylmethionine decarboxylase activity was si ficantly ($p < 0.05$) higher in the kidneys of castrated mice than in normal mice.

The changes in spermidine and spermine concentrations after treatment with testoster appeared to parallel the alteration in RNA concentration. This is interesting with res to the numerous reports indicating a relationship between polyamines and nucleic acids.

The rat ventral prostate gland is another organ showing responsivness to castration androgen treatment (Pegg *et al* 1970). However the changes in polyamine metabolism, a gonadectomy or androgen treatment, differ considerably from those in the kidney Am differences the most outstanding ones are the contrasts in enzyme activities. The prost ornithine decarboxylase activity was increased only a few times after testosterone adminis tion whereas S-adenosylmethionine decarboxylase activity was enhanced tenfold. The ferences are difficult to explain, but it should be kept in mind that the growth of the ven prostate after androgen treatment is a combination of hypertrophy and hyperplasia distinct from the kidney

Due to hypertrophy after testosterone administration the mouse kidney appear valuable organ in studying the importance of polyamines in RNA metabolism.

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Time course of light induced changes in pilocarpine sensitivity of rat iris

By

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Abstract

CLAESSON, H and E. BÁRÁNY *Time course of light induced changes in pilocarpine sens of rat iris* Acta physiol scand 1978 *102* 394–398

Pigmented rats were kept under different light regimens for 1 20 days. Supramaximal light was ... response of the iris sphincter in enucleated eyes with their cornea intact was tested in vitro. Pilocarp 6 10^{-4} M was used as standard stimulus. In agreement with previous authors there as a mark ference between constant light treated (subsensitive) eyes and dark treated (supersensitive) ones. At pretreated to reach maximal sub- and supersensitivity and then shifted to the opposite regimen, t that reversal was a gradual process starting within a few hours. Full reversal had not been reac 72 h. The two curves of sensitivity vs time intersected around 4 h. The time constants of the two were approximately equal. Light regimens using intermittent light shifting every 1 3, 6 or 12 h pro mediate results.

Key words: Pilocarpine, iris, subsensitivity light time course

Bito and Dawson (1970) and Bito *et al* (1971) have shown that the sensitivity of t sphincter to cholinergic stimulation varies—whether tested in vitro or in vivo—wi environmental light intensity

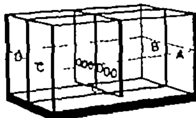
We have studied the time course for this modulation of sensitivity using the m method of Beaver and Riker (1962) also used by Bito

Our main findings are that 1) changes reach a steady state only after about a we the time course for rising and falling sensitivity are not markedly different.

Materials and Methods

Pigmented Lister Hooded male rats were from OLAC, Shaw f rm, Oxon, England and arrive fortnight. At time of arrival the weight of the rats was about 180 g. They were put into the animals q which had alternating light and darkness, shifting every 12 h, where they were allowed to adapt least one week. The time the rats spent in the animal house, before entering an experiment cou from one week to three weeks, which meant their weights varied from about 250 g to 325 g. This influence the results.

In vitro test of sensitivity
After treatment the rats, three at a time, were picked up at random out of their treatment group, g intraperitoneal injection of pentobarbital (veterinary mebumal) 60 mg/kg (A en) and deca



1 Diagram of the plexiglass chamber [illegible] its 2 compart[illegible], A and B, each of [illegible] hich in turn is partly divided by a [illegible] plexiglass [illegible] all, not reaching the floor and [illegible] ith 3 [illegible] The gas was bubbled by means of 2 needles deeply [illegible] into compartments C and D Compartment C [illegible] contained the contact thermometer The black plexi[illegible] tray was immersed in compartment A or B.

[illegible] minutes later The eyes were quickly enucleated, cleaned of extrabulbar tissue and the corneal epi[illegible] scraped off with [illegible] piece of razor blade. After [illegible] brief wash in incubation solution (see below), [illegible] was placed on [illegible] black plexiglass tray similar to that of Beaver and Riker with 6 hemispherical [illegible]. The tray was now immersed in Krebs-Henseleit bicarbonate solution, pH 7 4, bubbled with 93.5 [illegible] CO_2. The solution was contained in [illegible] clear plexiglass chamber (Fig. 1), [illegible] hich [illegible] as divided into [illegible] chambers, A–C and B–D, by [illegible] longitudinal wall allowing the use of two different concentra[illegible], or buffer [illegible] ith and without pilocarpine hydrochloride, at the same time. Temperature was held at [illegible] by means of [illegible] contact thermometer relay and two 15 W lamps inside [illegible] low heat capacity metal [illegible], in which the plexiglass chamber rested. Pupillary diameter was determined looking through the [illegible] using [illegible] stereomicroscope with an ocular micrometer, the magnification was approximately [illegible] There was [illegible] spotlight for the actual reading of the pupillary diameter no pupillary contraction was [illegible] by the light (cf [illegible]) Each of the chambers A–C and B–D were filled with 30 ml of buffer and the [illegible] with the eyes was placed in chamber A. After 30 min of incubation the pupillary diameter was mea[illegible] [illegible] control (D_c) and the tray was moved to chamber B, containing 6 [illegible] 10^{-4} mol/l of pilocarpine [illegible] concentration chosen after preliminary experiments) The drug solution was allowed to act for 30 min [illegible] in which the pupillary diameter (D_p) was measured, and the response was expressed as % miosis, [illegible] $(D_c - D_p)/D_c$. The percent miosis of the two eyes of [illegible] rat were averaged The 6 eyes run simultaneously [illegible] one test.

[illegible] treatment

[illegible] work [illegible] in the animal quarters and after [illegible] few hours of light in the morning, rats [illegible] were [illegible] picked up and put into clear plastic (Macrolon) cages (12 5 [illegible] 21 [illegible] 16 cm) with [illegible] wire mesh lid, [illegible] three rats in each cage. The bedding material consisted of wood shavings, and was changed 2 3 [illegible] week. The diet was composed of the same pellets as used in the animal house, R3 (Astra-Ewos, [illegible], Sweden) One to four Macrolon cages were now placed into specially designed light treatment [illegible]. These boxes, made out of hard board (bottom 75 [illegible] 75 and height 100 cm), consisted of an upper [illegible] lower part. The inside was painted white. The boxes were lighttight but not airtight. In the upper [illegible] 20 W/30 R (Osram L warmwhite) timer controlled fluorescent tubes were mounted. One of these [illegible] sufficient to give [illegible] effects there was no difference in degree of subsensitivity obtained if two [illegible] used The temperature in the cages was 22 ± 1°C.

Protocol of light treatment

[illegible] series, 1-4, were run The number of rats subjected to one specific light treatment was at most [illegible] In series 1–3 several light regimens were simultaneously run. In these series rats from different [illegible] were run in the same test. Other rats of the same group remained in the treatment boxes, thus [illegible] different time in the treatment boxes before sacrifice. The rats were exposed to the ambient light of the laboratory at most 30 min after removal from the treatment box.

Series 1. 48 rats were picked up from the animal house and 20 of them [illegible] were put into two light treat[illegible] boxes with constant light and the remainders were placed in another 2 treatment boxes, kept dark. 24 h later the first two rats were killed, one lighttreated, the other darktreated. Then, after 3 to 16 days [illegible] of treatment the rest of the rats were killed, equal numbers of animals from light and dark boxes [illegible] on any one day In series 2, six different light treatments were simultaneously used: Alternating light and darkness shifting every hour or every 3, 6 and 12 h, and also continuous light and darkness. [illegible] were put in the different boxes. After 6–30 days of treatment the animals were taken, always [illegible] from constant light and/or darkness, with alternating light and darkness treated ones in the same test.

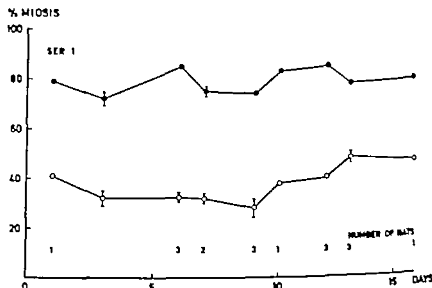


Fig. – Ser 1 The effects of continuous darkness (●) and light (O) on the sensitivity (miotic respo rat eyes. The points represent the main response in the number of rats shown in the lower part of the Vertical bars indicate ±S.E. If this is less than 1 it is hidden in the circles.

Series 3 Two groups of rats, 33 and 28 respectively were pretreated with continuous light and da for 9 days (216 h) before being shifted to the opposite regimen At time zero in their new regimen were sacrificed and the rest of them after 3–72 h of the new treatment.

Serie 4 This time 70 rats were put into constant light only and killed after 9–13 days of treatm

Results

The results from *series 1* showed a distinct difference in sensitivity of the iris sphinct tween constant light and dark treated animals (Fig. 2) This difference in miosis was already after only 24 h of treatment, and did not change appreciably as time for trea was prolonged.

In *series 2* the light treated rats showed a decrease in sensitivity as number of treat days increased above 6, in the dark treated ones, the miosis was almost constant durin expt. possibly because it was so near maximum (Fig. 3). Alternating light and dar produced a sensitivity that was almost independent of treatment duration above 6 d well as of the various cycle lengths and all animals showed a degree of miosis interme between that of light and dark treated ones.

Series 3 demonstrated that it took more than 72 h for full reversal of the light or dark induced changes. Reversal was a gradual process and the two curves intersected at about (Fig. 4).

Series 4 was run as a complement of series 1 and 2. All rats, independent of treat time, 9–13 days showed approximately the same miotic response, a response which within the range of the previous results from series 1 and 2.

A few expts. have further been run to examine the effect of even longer treatment. 3 days of treatment sensitized the animals just as much as 10–15 rats).

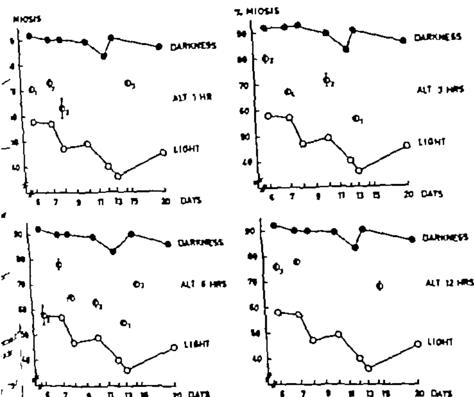


Fig. 3. Ser. 2 compares the effects of constant light (○), darkness (●), and alternating light and darkness treatment (◐). Each circle represents the mean response in the number of rats shown to the right of the circle representing alternating light and darkness. For darkness treated ones (cf. Fig. 4) the points represent [illegible], 2, 1, 1, 2, 1 rats and for light treated 2, 4, 2, 2, 3, 1 rats. Vertical bars indicate ± S.E. (as in Fig. 2). Dotted lines represent the mean effects of the other alternating treatments shown in the other panels. The points representing continuous light and darkness are shown with their S.E. as in Fig. 4.

Discussion

The pilocarpine concentration $6 \cdot 10^{-4}$ mol/l was chosen so as to make the sub- and the supersensitivity responses lie symmetrically on the dose-response curve as in series 1. For unknown reasons, the degrees of miosis in the later series were higher and the range of variation no longer centered around 50% miosis. This introduces a bias into the comparison between the time courses of recovery from darkness and light (series 3): one underestimates the rate of change in the initial parts of the recovery from dark treatment. Despite this difficulty there is a striking similarity in the temporal development of supersensitivity and in that of subsensitivity. The changes progress slowly and steady state seems to be reached only after about a week. But it is not certain that the degree of sub- or supersensitivity reached is determined by the effector organ proper, the smooth muscle of the sphincter. Excitation of the sphincter in our experiments is by way of the pupillary light reflex and it could well be that other stations in the reflex arc also suffer changes during continuous light or darkness, subsensitivity during continuous activity and maybe even spontaneous activity after

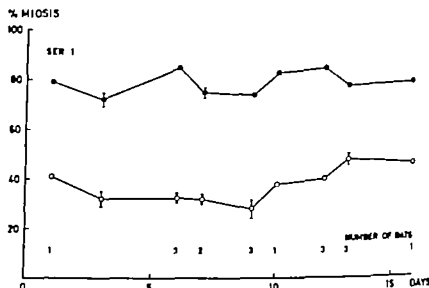


Fig. 2. Ser 1 The effects of continuous darkness (●) and light (○) on the sensitivity (miotic respons rat eyes. The points represent the main response in the number of rats shown in the lower part of the fi Vertical bars indicate ± S.E. If this is less than 1 % it is hidden in the circles.

Series 3 Two groups of rats, 33 and 28 respectively were pretreated with continuous light and dark for 9 days (216 h) before being shifted to the opposite regimen. At time zero in their new regimen 4 were sacrificed and the rest of them after 3–72 h of the new treatment.

Series 4 This time 20 rats were put into constant light only and killed after 9–13 days of treatment

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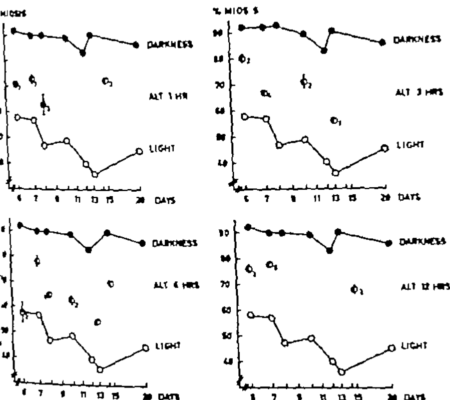


Fig. 5. Ser. 2 compares the effects of constant light (○), darkness (●), and alternating light and darkness treatment (◐). Each circle represents the mean response in the number of rats shown to the right of the circle representing alternating light and darkness. For darkness treated ones (cf. Fig. 4) the points represent 1, 2, 1, 1, 2, 1 rats and for light treated 2, 4, 2, 2, 3, 1 rats. Vertical bars indicate ±S.E. (as in Fig. 2). Dotted lines represent the miotic effect of the other alternating treatments shown in the other panels. The points representing continuous light and darkness are shown with their S.E. as in Fig. 4

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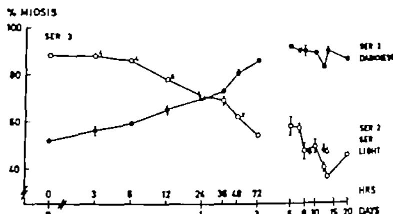


Fig. 4 *Ser 2* is shown here to make it possible to compare ser 2, 3 and 4, see further the legend of Fi *Ser 3* shows the responses in rats pretreated with 9 days of constant light and darkness and then sh to the opposite regimen. The same symbols for different treatments are used as in Fig. 2 and 3, also vertical bars indicate ±S.E. and the number of rats is indicated to the right of the dots. Note the i section between the curves t about 4 h. *Ser 4* is rats that have been light treated for 9 12 and 13 (Δ). The triangles represent 9 6 and 5 rats.

prolonged total darkness. The changes observed in vitro thus are the minimum that con excitation or constant lack of excitation could cause and the apparent appearance of a st state after about a week does not necessarily indicate that the smooth muscle regulate sensitivity over only this span of time or only to the extent actually seen.

The loss and the recovery of sensitivity seemed to occur along similar time courses. accords with the findings in series 2, where intermittent symmetrical lighting schedules used. Intermittent light causes intermediate degrees of sensitivity This would not be expe if the time course of recovery from darkness were very different from the time cours recovery from light On the whole, our findings are compatible with the assumption of (1971) that the receptor density on the smooth muscle cells is regulated and with the fur assumption that the receptor population involved has a half-life of approximately 1 2 d Strong excitation would prevent the formation of new receptors without accelerating decay of already formed ones, lack of excitation would accelerate the formation of receptors again without affecting the removal of already formed ones. This concept expl the similarity in the time course of sensitivity increase and decrease, it is the time cons of receptor removal which governs the time constant of both observable processes.

Bito (1971) has pointed out that choline esters and pilocarpine seem to act on diffe populations of receptors. Since the present expts. were all done with pilocarpine as agonist, it is too early to predict if the time course for the other group is similar

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Inhibition of vasopressin-release during developing hypernatremia and plasma hyperosmolality: An effect of intracerebroventricular glycerol

By

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Abstract

Olsson, K., F. Fyhrquist, B. Larsson and L. Eriksson. *Inhibition of vasopressin-release during developing hypernatremia and plasma hyperosmolality: An effect of intracerebroventricular glycerol.* Acta physiol. scand. 1978. *102*. 399–409.

In well-hydrated goats prolonged (3 h, 0.02 ml/min) intracerebroventricular (IVT) infusion of 0.35 M glycerol depressed the plasma vasopressin level during the entire infusion period which resulted in a continuous water diuresis outlasting the infusion by about 20 min. Since no compensatory drinking occurred during the sustained water diuresis it gradually induced pronounced dehydration (loss of > 1 liter of total body water causing 3% increase in plasma [Na^+] and osmolality). The same degree of dehydration was in other experiments induced by water deprivation. It then caused a 5-fold increase in plasma vasopressin level.

Corresponding IVT infusions of 0.35 M d-glucose depressed plasma vasopressin level only during the first half of the 3 h infusion period. Consequently the resulting water diuresis was transient and subsided when the glucose infusion was finished.

Plasma renin activity increased during the IVT glycerol infusion and during water deprivation, but was only slightly influenced by IVT glucose. Both IVT glycerol and glucose decreased renal sodium excretion.

The possibility is discussed that the pronounced ability of IVT glycerol to depress the vasopressin release is not only due to dilution induced reduction of CSF [Na^+], but also to an influence of glycerol on choroidal and/or transependymal Na^+-transporting mechanisms.

Sodium sensitive receptors close to the anterior wall of the third cerebral ventricle seem to play an essential role in the regulation of antidiuretic hormone (ADH) release and water intake (cf. Andersson and Olsson 1977). Among the evidence for this concept are the observations that lowering of the [Na^+] of the cerebrospinal fluid (CSF) by intracerebroventricular (IVT) infusions of non-electrolyte solutions induce a water diuresis in normally hydrated, normovolemic goats (Eriksson 1974, 1976), and attenuate dehydrative drinking (Olsson 1975). It was recently found that 1 h IVT infusions of glycerol are much more effective in these respects, and in lowering CSF [Na^+], than the corresponding infusions of

d-glucose (Olsson, Larsson and Liljekvist 1976) However no quantitative determin of the effect on the ADH release (plasma Arginine Vasopressin – AVP) has been ma previous experiments involving IVT infusions of non-electrolyte solutions. This made interest in the present study to follow the effects on water balance and AVP-release of longed (3 h) IVT glycerol and d-glucose infusions.

A positive correlation between CSF [Na^+] and renal sodium excretion has been onstrated in several species (*cf* Andersson 1977), whereas changes in CSF [Na^+] ap to have the reverse effect on plasma renin activity (PRA) (Mouw and Vander 1970, M *et al* 1974 Eriksson and Fyhrquist 1976) Therefore, the present study was extended t volve also determinations of PRA and renal electrolyte excretion.

Methods

Animals 5 female goats (b.wt. 30–45 kg) were used They were housed in individual metabolism cage free access to water except in dehydration experiments. The goats received hay *ad lib.* and were each noon given 6 g of NaCl in 300 g of commercial grain mix.

Implantations and infusions int the lateral cerebral ventricle All goats had a three-cannula system manently impla ted into one of the lateral ventricles with the ventricular outlet near the foramen of M The cannula material was platinum-iridium in order to minimize foreign body reaction. The constru of the cannula system and the infusion technique ha e been described earlier (Åkerlund, Andersson Olsson 1973). Major advantages of the infusion technique employed were that no dead space ad to the system, and that the infusions mixed freely with the ventricular CSF and that the infusions be started and stopped without the animals being aware of it. Furthermore, samples of CSF could be obtained for analyses la the cannula system used.

Sampling f CSF Collection of CSF samples were made as previously described (Olsson *et al.* before, and 20 min after the infusions. About 0.5 ml of CSF was collected in glass tubes each tim analyses of Na K+ and osmolality The removal of this relatively small amount of CSF did not cause visible disturbance f the animals.

Sampling of blood Blood samples were obtained la Braunule cannula inserted into the jugular Blood for hormone radioimmunoassay was collected ice-chilled centrifuge tubes with 0.3 M EDT anticoagulant. The blood was immediately centrifuged at +4°C nd the plasma was stored at −20°C radioimmunoassayed. Blood samples for determinations of plasma Na K and osmolality were colle in heparinized tubes.

Sampling of urine Urine was collected via a Foley catheter inserted into the urinary bladder

Anal tical techniques Radioimmunoassay for plasma AVP was performed with method previo applied t determination of human plasma AVP (Fyhrquist *et al* 1976 b). The antiserum used as gener in a rabbit using rginine-vasopressin as a hapten nd bovine thyroglobulin as a carrier Cross-reac with oxytocin (Sandoz, Basel) was < 0.03 ., with pressinoic acid (Ferring, Malmö) < 0.1 ., with C-term tripeptide of vasopressin (Ferring) < 0.01 %. Sequential saturation nd the use of the Sigma VII A preparation (Sigma, St Louis, Missouri) for standards and preparation of ^{125}I-labelled tracer increa the assay sensitivity t 0.2 pg/ml. Recovery of synthetic AVP added to pooled goat plasma as 67–9 in the low range (2.8 pg/ml added) and 82 105 % in the high range (28 pg/ml added).

Radioimmunoassay of PRA was modified for assay of goat PRA according to Fyhrquist *et al.* (197 The optimal pH of angiotensin I generation in goat plasma was found to be 6.2. Therefore, samples w incubated at pH 6.2, in the presence of OH-quinoline 3.5 mM, nd Na-EDTA 15 mM There as no cha of pH during the 2 h incubation at +37°C. Radioimmunoassay sensitivity was 2.5 pg angiotensin I (2 S deviation from zero dose of unlabelled). I terassay variation was 8.5 ., intra-assay ariation 5.8 .

CSF plasma and urine Na and K were measured by internal standard flame photometry using IL 343 flame photometer An Adv Instruments Inc. osmometer was used for determination of osmolality of these fluids. All values presented represent mean nd S.E.

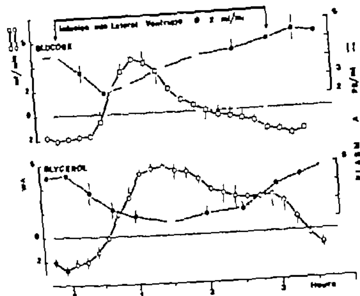


Fig. 1. *Above:* Transient depression of the basal AVP-release during IVT infusions of 0.35 M glucose. Consequently, the induced water diuresis subsides before end of the infusion period. Number of expts. = 10, [illegible] 4. *Below:* More pronounced and sustained inhibition of the AVP-release during IVT infusions of 0.35 M glycerol. Thus, the resulting water diuresis lasts throughout the infusion period. Number of expts. = [illegible] 4. Symbols represent means, vertical bars S.E.

Results

Effects of 3 h IVT glycerol and glucose infusions

[illegible] of AVP-release and thirst: The IVT infusion of *glycerol* invariably induced a [illegible] water diuresis in the non-hydrated, normovolemic goat. From pre-infusion [illegible] values, renal free water clearance (C_{H_2O}) turned positive 30–40 min after the onset [illegible] the infusion and reached maximal level (5.6 ± 0.5 ml/min) 30 min later. Thus far the effect [illegible] identical to that earlier obtained in response to the 1 h infusion (Olsson *et al.* 1976). However due to the present prolonged IVT administration the water diuresis was sustained [illegible] for about 3 h, although with some reduction in magnitude (C_{H_2O} about 3 ml/min) after 2 h [illegible] infusion (Fig. 1). At this time the animals had become markedly dehydrated as indicated by a rise in plasma [Na] from 148.0 ± 0.7 to 156 ± 0.7 mmol/l. Determinations of plasma AVP showed that this water diuresis was associated with a reduction of plasma AVP levels. Plasma AVP started to decline immediately after the onset of the glycerol infusion, reached [illegible] value at the time of maximal water diuresis and remained below pre-infusion level as long as renal C_{H_2O} was positive (Fig. 1). Thus, unlike in the water deprivation experiments [illegible] below), there was no correlation between plasma [Na] and plasma AVP values during IVT glycerol infusions (Fig. 2).

Initially the reduction of the plasma AVP level and the development of the water diuresis followed a similar pattern when, instead of glycerol, d-*glucose* was infused for 3 h into the lateral ventricle. However the IVT administration of glucose was much less effective in

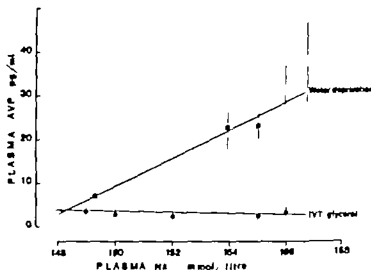


Fig. 2. *Squares.* Highly signific correlation (y 403 12 32 r = 0.65, p < 0 001) between plas AVP values and plasma [Na⁺] du ing dehydration due to water d privation. Number of expts. goats = 4. *Dots:* Lack of such a relation during correspond stages of dehydration induced) IVT glycerol infusions (y 15 17 0.08x, r = −0.20). Number expts. = 11 goats 4. Symb represent mean, vertical bars SI

maintaining a depression of plasma AVP Consequently in these experiments, the wat diuresis subsided within 2 h of IVT infusion At this stage plasma AVP had returned t pre-infusion level (Fig. 1)

The IVT administration of glycerol and glucose did not distress the goats or cause be havioural reactions. They were alert and continued to eat and ruminate intermitting during the entire infusion periods. However their urge to drink in response to dehydratio was obviously inhibited. In spite of gradually developing dehydration due to excessi urinary water loss, none of the animals drank during the IVT infusions. One of the goats responding to IVT glycerol with the most intense water diuresis, regularly compensated t water loss about 1 h post-infusion Compensatory drinking was even more delayed in th other animals.

Increase in plasma [Na⁺] and osmolality The water diuresis induced by the 3 h IVT in fusion of *glycerol* resulted in a 620 ± 120 ml net loss of "free" water and a total fluid loss o 1 090 ± 180 ml (= about 4% reduction of total body water).

As a result plasma [Na] and osmolality increased by 5%. The animals lost considerabl less water due to the IVT *glucose* infusions. Hence, in these experiments the increase i plasma [Na] and osmolality was limited to 3%. Plasma [K] was not significantly affecte by any of the infusions. The data regarding these changes in blood plasma composition a presented in Table I

Effects on CSF [Na], [K] and osmolality Samples of CSF were taken via the sam cannula system which was used for the IVT infusions. Therefore, no samples could be ob tained during the infusion periods and the post infusion sample was not taken until 20 mi later *i.e.* at a time when the infused solution by all probability had become equilibrate with the CSF of the lateral ventricle. It has been reported previously (Olsson *et al.* 197 that IVT infusion of *glycerol* markedly depresses CSF [Na]. This observation was confirme in the present study The 3 h IVT infusion of glycerol induced a fall in CSF [Na] from th pre infusion level of 151 ± 1 to 117 ± 5 mmol/l (n = 9) 20 min postinfusion The fact that th glycerol solution was slightly hypertonic (0.35 M) explains the simultaneous increase i

TABLE I. Changes in plasma [Na+], [K+], and osmolality during IVT glycerol (0.35 M) and IVT d-glucose (0.35 M) infusions. The infusion period was 3 h and the rate of the infusion 0.02 ml/min. Note the marked increase in plasma [Na+] and osmolality at the end of IVT glycerol infusions. Glycerol: n = 11, goats = 4; glucose: n = 10, goats = 4

	Pre-inf.	Infusion							20 min post-inf.
		20 min	40 min	60 min	90 min	120 min	150 min	180 min	
Na mmol/l									
Glycerol	148.0±0.7	149.1±0.7	150.0±0.6	152.0±0.6	152.7±0.7	156.0±0.7	154.9±0.9	156.0±0.8	155.9±1.2
Glucose	149.0±0.9	149.4±0.7	150.1±1.0	150.6±1.0	153.0±1.1	153.3±1.1	153.5±1.2	151.6±1.1	152.1±1.5
K mmol/l									
Glycerol	4.2±0.1	4.0±0.1	3.9±0.1	4.1±0.1	4.2±0.1	4.1±0.1	4.2±0.1	4.2±0.1	4.0±0.1
Glucose	4.1±0.1	3.9±0.1	3.9±0.1	3.9±0.1	4.0±0.1	3.9±0.1	3.9±0.1	4.0±0.1	3.9±0.1
mosm/kg									
Glycerol	296±1	297±1	298±1	300±2	302±1	307±2	306±2	309±2	309±3
Glucose	297±1	299±1	300±1	302±1	304±2	306±1	307±1	304±2	305±2

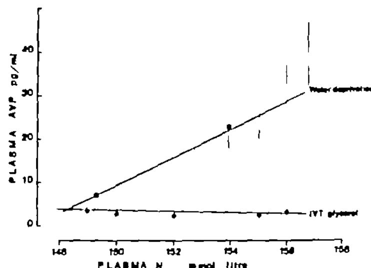


Fig. 2. *Squares* Highly signific correlation (y -43.82) r = 0.65, p < 0.001) between plas AVP values and plasma [Na⁺] d ing dehydration due to water deprivation. Number of expts goats = 4. *Dots* Lack of such c relation during correspond stages of dehydration induced IVT glycerol infusions (y 15 0.08x, r 0.20). Number expts = 11 goats 4 Symb represent mean, vertical bars SE

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	Pre-inf	Infusion							20 min post-inf
		20 min	40 min	60 min	90 min	120 min	150 min	180 min	
Na mmol/l									
Glycerol	148.0±0.7	149.1±0.7	150.0±0.6	152.0±0.6	152.7±0.7	156.0±0.7	154.9±0.9	156.0±0.8	155.9±1.2
Glucose	149.0±0.9	149.4±0.7	150.1±1.0	150.6±1.0	153.0±1.1	153.3±1.1	153.5±1.2	151.6±1.1	152.1±1.5
K mmol/l									
Glycerol	4.2±0.1	4.0±0.1	3.9±0.1	4.1±0.1	4.2±0.1	4.1±0.1	4.2±0.1	4.2±0.1	4.0±0.1
Glucose	4.1±0.1	3.9±0.1	3.9±0.1	3.9±0.1	4.0±0.1	3.9±0.1	3.9±0.1	4.0±0.1	3.9±0.1
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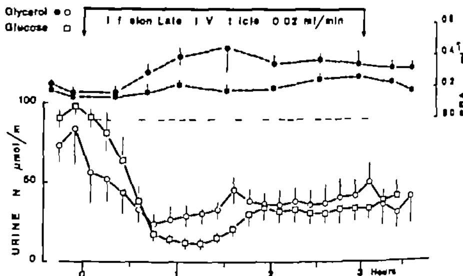


Fig. 3 *Filled symbols.* Increase in PRA during IVT infusions of glycerol (0.35 M) compared to the negligible effect of corresponding glucose infusions. *Open symbols* Marked depression of renal Na excretion during both kinds of IVT infusion. Glycerol Number of expts. = 11 goats = 4. Glucose: Number of expts goats = 4 Symbols represent mean, vertical bars S.E.

CSF osmolality from 298 ± 2 to 310 ± 3 mosm/kg. CSF [K] decreased slightly (2.8 + 0.0 to 2.7 ± 0.0 mmol/l). In comparison the IVT *glucose* infusion only depressed CSF [Na] to 134 ± 2 mmol/l (n = 8), while the osmolality increased to 305 ± 1 mosm/kg. The CSF [K remained unchanged 20 min after the infusion of glucose.

Increase in PRA The IVT infusion of *glycerol* was accompanied by an increase in PRA which rose from 0.25 ± 0.04 ng ml h prior to the infusion to a peak level of 0.45 ± 0 ng ml⁻¹ h after 90 min of infusion (Fig. 3) Thus, PRA reached its highest value at the time when plasma AVP was at its lowest. PRA then declined to about 0.35 ng ml⁻¹ h and this level was maintained throughout the infusion period Only a minor increase in PRA was observed during the IVT *glucose* infusion (Fig. 3) PRA rose from 0.18 ± 0.03 to 0.25 ± 0.03 ng ml h within 60 min and continued to fluctuate around this level throughout the infusion period. It had returned to the pre infusion value 40 min after the infusion.

Decrease in renal Na and K excretion. The infusions of both glycerol and glucose caused a reduction in renal Na excretion (Fig. 3) and to some extent also in renal K excretion. A decrease in Na$^+$ excretion was seen within the first 30 min which became significant after 50 min After some rise, the Na excretion was maintained at about 50% of the pre-infusion level throughout the infusion and during the 30 and 40 min post-infusion observation periods. Renal K excretion showed a similar pattern but the reduction was less pronounced (about 25%).

Effects of water deprivation

The fact that IVT glycerol markedly depressed plasma AVP in spite of developing dehydration made it of interest to study the effects of dehydration to a similar extent under conditions when the CSF composition was allowed to follow the blood plasma changes. In these control experiments drinking water was withheld, and blood, CSF (one animal)

Changes in plasma parameters during 55 h of water deprivation and after rehydration. No. of goats = 4.

	AVP pg/ml	PRA ng ml^{-1} h^{-1}	Na mmol/l	K mmol/l	Osmolality mosm/kg
r water d (h)					
	7.28 ± 1.78	0.12 ± 0.02	149.3 ± 0.3	4.2 ± 0.2	293 ± 1
	6.78 ± 1.48	0.14 ± 0.02	149.7 ± 0.1	4.2 ± 0.2	294 ± 1
	9.63 ± 1.28	0.21 ± 0.02	151.4 ± 0.2	4.4 ± 0.1	298 ± 1
	15.53 ± 2.75	0.19 ± 0.04	152.7 ± 0.7	4.2 ± 0.1	299 ± 1
	23.10 ± 5.92	0.23 ± 0.03	153.9 ± 0.4	4.2 ± 0.2	303 ± 1
	23.00 ± 3.36	0.35 ± 0.04	155.2 ± 0.8	3.9 ± 0.1	305 ± 1
	31.43 ± 6.42	0.25 ± 0.04	155.9 ± 0.8	3.9 ± 0.1	307 ± 3
	37.95 ± 10.83	0.22 ± 0.04	156.7 ± 0.9	4.1 ± 0.1	310 ± 3
r n (h)					
	8.50 ± 1.37	0.36 ± 0.03	152.8 ± 1.0	3.7 ± 0.1	299 ± 3
	6.55 ± 0.84	0.31 ± 0.05	150.2 ± 1.1	4.1 ± 0.1	298 ± 2
	7.00 ± 0.85	0.33 ± 0.05	150.1 ± 1.0	4.0 ± 0.1	294 ± 3

…e samples were taken at intervals until plasma Na reached 156 mmol/l and plasma …ty 309 mosm/kg (= the same mean values as recorded at the termination of the …cerol infusions). These values were reached after 55 h of water deprivation, and then …als were given free access to water. The gradual changes in the composition of the …lasma during the 55 h deprivation period, and the effects of rehydration are shown in …. In the single animal studied, CSF [Na] had increased from 151 to 161 mmol/l and …molality from 295 to 312 mosm/kg during the 55 h of water deprivation. Ninety …r that the animal had satisfied its thirst, the corresponding CSF values were 156 … and 305 mosm/kg.

…lation of the AVP-release: As could be expected from similar studies in man (Robert… Arbar 1976), plasma AVP gradually rose during water deprivation concomitant with …rise in plasma [Na] and osmolality. In Fig. 2 is shown the highly significant relation … increases in plasma [Na] and the rises in plasma AVP which were observed during …ation induced by water deprivation. For comparison is shown the total lack of such …lation at corresponding stages of dehydration during the 3 h IVT infusion of glycerol.

…ase in PRA. PRA rose during the first 50 h of water deprivation (Table II). It then …d during the remaining 5 h of continued dehydration. However 30 min after that …mals had drunk to satiety there was a steep rise in PRA, and this high level was …ined also in samples taken 60 and 90 min after rehydration.

…l Na^+ and K^+ excretion: Dehydration induced by water deprivation did not seem to … the renal Na^+ excretion which remained between 50 and 125 µmol/min for the whole …eriod. The renal K excretion fell from a level of about 250 µmol/min during the first …o about 130 µmol/min during the last 5 h of water deprivation. The K excretion …ned at the lower level also for at least 90 min after rehydration. It appears probable …duced food consumption was the reason for the depressed renal K excretion towards …d of the dehydration period.

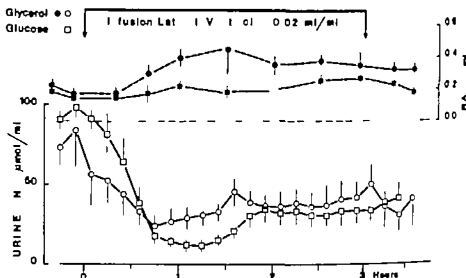


Fig. 3 *Filled symbols.* Increase in PRA during IVT infusions of glycerol (0.35 M) compared to the negligible effect of corresponding glucose infusions. *Open symbols:* Marked depression of renal Na^+ excretion during both kinds of IVT infusion Glycerol Number of expts. = 11 goats = 4 Glucose Number of expts goats = 4 Symbols represent mean, vertical bars S.E.

CSF osmolality from 298 ± 2 to 310 ± 3 mosm/kg. CSF [K] decreased slightly (2.8 ± 0.0 to 2.7 ± 0 0 mmol/l). In comparison the IVT *glucose* infusion only depressed CSF [Na] to 134 ± 2 mmol/l (n = 8) while the osmolality increased to 305 ± 1 mosm/kg. The CSF [K remained unchanged 20 min after the infusion of glucose.

Increase in PRA The IVT infusion of *glycerol* was accompanied by an increase in PRA which rose from 0 25 ± 0 04 ng ml h prior to the infusion to a peak level of 0.45 ± 0. ng ml h after 90 min of infusion (Fig. 3) Thus, PRA reached its highest value at the time when plasma AVP was at its lowest PRA then declined to about 0.35 ng ml^{-1} h and this level was maintained throughout the infusion period. Only a minor increase in PRA was observed during the IVT *glucose* infusion (Fig. 3). PRA rose from 0.18 ± 0 03 to 0.25 ± 0 03 ng ml h within 60 min and continued to fluctuate around this level throughout the infusion period It had returned to the pre-infusion value 40 min after the infusion.

Decrease in renal Na and K excretion The infusions of both glycerol and glucose caused a reduction in renal Na excretion (Fig. 3), and to some extent also in renal K^+ excretion A decrease in Na^+ excretion was seen within the first 30 min which became significant after 50 min After some rise, the Na excretion was maintained at about 50 % of the pre-infusion level throughout the infusion and during the 30 and 40 min post infusion observation periods. Renal K^+ excretion showed a similar pattern but the reduction was less pronounced (about 25 %)

Effects of water deprivation

The fact that IVT glycerol markedly depressed plasma AVP in spite of developing dehydration made it of interest to study the effects of dehydration to a similar extent under conditions when the CSF composition was allowed to follow the blood plasma changes. In these control experiments drinking-water was withheld and blood, CSF (one animal)

…LE II. Changes in plasma parameters during 55 h of water deprivation and after rehydration. No. of …pats 4.

	AVP pg/ml	PRA ng·ml⁻¹·h⁻¹	Na mmol/l	K mmol/l	Osmolality mosm/kg
…me after water …thdrawal (h)					
	7.20 ± 1.70	0.12 ± 0.02	149.3 ± 0.3	4.2 ± 0.2	293 ± 1
	6.78 ± 1.48	0.14 ± 0.02	149.7 ± 0.1	4.2 ± 0.2	294 ± 1
	9.63 ± 1.28	0.21 ± 0.02	151.4 ± 0.2	4.4 ± 0.1	298 ± 1
	15.57 ± 2.75	0.19 ± 0.04	152.7 ± 0.7	4.2 ± 0.1	299 ± 1
	23.10 ± 5.92	0.23 ± 0.03	153.9 ± 0.4	4.2 ± 0.2	303 ± 1
	23.00 ± 3.36	0.35 ± 0.04	155.2 ± 0.8	3.9 ± 0.1	305 ± 1
	31.43 ± 6.42	0.25 ± 0.04	155.9 ± 0.8	3.9 ± 0.1	307 ± 3
5	37.95 ± 10.13	0.22 ± 0.04	156.7 ± 0.9	4.1 ± 0.1	310 ± 3
…me after …hydration (h)					
5	8.50 ± 1.37	0.36 ± 0.03	152.8 ± 1.0	3.7 ± 0.1	299 ± 3
5	6.55 ± 0.84	0.31 ± 0.05	150.2 ± 1.1	4.1 ± 0.1	298 ± 2
	7.00 ± 0.85	0.33 ± 0.05	150.1 ± 1.0	4.0 ± 0.1	294 ± 3

…al urine samples were taken at intervals until plasma Na reached 156 mmol/l and plasma …molality 309 mosm/kg (= the same mean values as recorded at the termination of the …VT glycerol infusions). These values were reached after 55 h of water deprivation, and then …e animals were given free access to water. The gradual changes in the composition of the …lood plasma during the 55 h deprivation period, and the effects of rehydration are shown in …able II. In the single animal studied, CSF [Na] had increased from 151 to 161 mmol/l and …SF osmolality from 295 to 312 mosm/kg during the 55 h of water deprivation. Ninety …min after that the animal had satisfied its thirst, the corresponding CSF values were 156 …mmol/l and 305 mosm/kg.

…timulation of the AVP-release. As could be expected from similar studies in man (Robert…son and Athar 1976), plasma AVP gradually rose during water deprivation concomitant with …the increase in plasma [Na^+] and osmolality. In Fig. 2 is shown the highly significant relation …between increases in plasma [Na] and the rises in plasma AVP which were observed during …dehydration induced by water deprivation. For comparison is shown the total lack of such …a correlation at corresponding stages of dehydration during the 3 h IVT infusion of glycerol.

Increase in PRA. PRA rose during the first 50 h of water deprivation (Table II). It then …declined during the remaining 5 h of continued dehydration. However, 30 min after that …the animals had drunk to satiety there was a steep rise in PRA, and this high level was …maintained also in samples taken 60 and 90 min after rehydration.

Renal Na^+ and K excretion. Dehydration induced by water deprivation did not seem to …affect the renal Na excretion which remained between 50 and 125 µmol/min for the whole …55 h period. The renal K excretion fell from a level of about 250 µmol/min during the first …50 h to about 130 µmol/min during the last 5 h of water deprivation. The K excretion …remained at the lower level also for at least 90 min after rehydration. It appears probable …that reduced food consumption was the reason for the depressed renal K excretion towards …the end of the dehydration period.

Discussion

A continuing uncompensated loss of body water leads to absolute dehydration. This condition is characterized by hyperosmolality and reduced volume of all body fluid compartments and by elevated extracellular [Na]. The main compensatory mechanisms brought into play during absolute dehydration are increased release of ADH and thirst with the dual aim to prevent further unnecessary renal waste of water and to restore the water deficit by drinking. It has become generally accepted that the interaction between an "osmotic" and a "volume" regulation governs mammalian ADH-release and water intake. Verney's (1947) ingenious studies conclusively demonstrated that cerebral receptors in the hypothalamic region are involved in the regulation of the ADH-release and implied that these osmoreceptors primarily are excited by changes in the composition of the extracellular fluid which lead to a reduction of the receptor volume. This osmoreceptor concept was extended to involve also the regulation of water intake when it was shown that injections of hypertonic NaCl into the anterior hypothalamus elicited excessive drinking in water-replete animals (Andersson 1953). What concerns the "volume" control of water balance, considerable evidence favours the concept that distension receptors in the cardio-vascular system exert a reflex inhibitory tonus of the neurohypophyseal release of ADH (cf Gauer Henry and Behn 1970). In addition the renal renin angiotensin system appears to form an important humoral link in the "volume" regulation of thirst and ADH release (cf Fitzsimons 1972).

Paradoxically the osmoreceptor concept eliminates in a strict sense the distinction between "osmotic" and "volume" control of water balance since hypothalamic "osmoreceptors" are thought to monitor intracellular fluid volume. Studies performed predominantly in the goat over the past several years strongly suggest that, instead of osmoreceptors juxtaventricular sodium sensitive receptors play an essential role in the regulation of ADH-release and thirst (cf Andersson and Olsson 1977). This novel idea reverts to a distinction between a regulation influenced by a solute composition of the body fluids on the one hand and "volume" (=extracellular) regulation on the other On the whole, the experiments reported here provide further support for this idea. In the present study the same goats were dehydrated to a similar extent in two different ways a) by water deprivation, and b) by rapidly losing a large amount of body water in response to IVT glycerol In the first case dehydration induced the appropriate compensatory mechanisms (thirst and accelerated ADH release), in the second case not. It clearly demonstrates that even pronounced changes in the blood of the kind which via cerebral receptors normally stimulate ADH-release and thirst can be made ineffective by IVT infusions of glycerol. This indirectly supports the view (cf Andersson 1971) that the choroid plexa/CSF is a route by which blood-borne stimuli affect cerebral mechanisms involved in the control of water balance. At the same time, however the prolonged IVT infusions of glycerol and glucose indicate that the receptors are also affected more directly by the composition of the blood plasma. In spite of maintained subnormal [Na] of the CSF the plasma AVP had started to rise again towards the end of the infusion period. It suggests that intense blood-borne stimuli and/or hypovolemic factors may overcome inhibitory influences from the CSF Ablation experiments (Andersson, Leksell and Lishajko 1975) suggest that the cerebral receptors whic te ADH-release

al thirst predominantly are located in the surroundings of the anterior portion of the third ventricle. Since circumventricular structures there are devoid of a blood brain barrier (*cf* Weindl and Joynt 1972), sensory elements in that area may be easily accessible to both blood- and CSF-borne stimuli. The experiments reported here confirm and extend the previous observations (Olsson *et al.* 1976) that IVT glycerol lowers CSF [Na] and inhibits ADH-release and thirst more effectively than does IVT glucose. It is in agreement with the concept that a juxtaventricular sodium sensitive mechanism is involved in the control of water balance. However it leaves open questions such as a) why do the two substances have such differing effects upon CSF [Na]? b) do factors other than mere reduced CSF [Na] contribute to the remarkable efficiency of IVT glycerol as inhibitor of ADH release and thirst? Choroidal Na K -ATPase activity is a prerequisite for normal Na transport from the blood to the CSF (Vates *et al.* 1964), and glycerol is an in vitro inhibitor of that enzyme system in electrophorus electricus (Mayer and Avi-Dor 1970) and in ox brain microsome (Albers and Koval 1972). Therefore, reduced entrance of Na into the CSF via the choroid plexus of the lateral ventricle may explain why CSF [Na] was 17 mmol/l lower after the IVT infusions of glycerol than after the corresponding glucose infusions. It appears less likely that choroidal Na -coupled glycerol transport out of CSF would have contributed to the observed difference, since transport of that kind would be expected to affect glucose (Bradbury and Brøndsted 1973) to at least the same extent as glycerol. However, a contributing factor may also have been glycerol-induced inhibition of periventricular enzymatic Na transport elsewhere than in the choroid plexa. Reduced pumping of Na out of cells surrounding the ventricles may have increased the net diffusional transport of Na from the CSF. If so, the effective stimulus of juxtaventricular sodium sensitive receptors could hardly be increased intracellular Na *per se*. It rather suggests that local Na K ATPase activity may be of crucial importance for excitation of "sodium receptors" involved in the control of water balance.

Some support for this very hypothetical speculation is provided by the observation that IVT infusions of deuterium (with NaCl added to isotonicity), like glycerol, effectively inhibit ADH-release and thirst in the goat (Leksell, Lishajko and Rundgren 1976). Deuterium has also been shown to inhibit Na K ATPase (Ahmed and Foster 1974).

Arginine vasopressin (AVP) is the endogenous ADH in the goat (*cf* Valtin, Stewart and Sokol 1974). That the radioimmunoassay method employed for the present AVP determinations (Fyhrquist *et al.* 1976 b) gives a reliable index of the neurohypophyseal ADH-release is evidenced by the highly significant relation between plasma [Na] and AVP values which was obtained during the developing dehydration induced by water deprivation (Fig. 2). Hence, this control study supports the assumption that the observed fall in plasma AVP during IVT infusion of glycerol and glucose was due to an inhibition of the basic ADH-release normally going on in the non-hydrated, normovolemic animal.

Artificially induced elevations of the CSF [Na] have been found to increase renal Na+ excretion in many species, which suggests that a juxtaventricular sodium sensitive system also operates in the regulation of renal Na output. In consistence with this idea is the observation in the anaesthetized dog (Mouw and Vander 1970) and the conscious sheep (Mouw *et al.* 1974) that perfusion of the cerebroventricular system with artificial CSF of low Na

reduces renal Na excretion. However in this respect previous 1 h IVT infusions of vario non-electrolyte solutions have given inconclusive results in the goat. In the nonhydrate animal such infusions were not found to induce any consistent change in renal electrolyt excretion (Eriksson 1974), whereas in the hydrated goat the 1 h IVT infusion of fructo caused a fall in the renal K excretion, indicating a decreased sodium load at the distal tubu in the kidney (Eriksson and Fyhrquist 1976). However in the present experiments a signif cant reduction in renal Na excretion was obtained in response to the IVT infusions c glycerol and glucose. It indicates that also in the goat a lowering of CSF [Na] affects rena Na output in negative direction, and supports the view that juxtaventricular sodiu sensitive receptors participate in the control of sodium homeostasis.

In previous studies it has been found that lowering of CSF [Na] causes a rise in PRA (Mouw and Vander 1970 Mouw *et al.* 1974) whereas IVT infusions of hypertonic NaC depress PRA (Eriksson and Fyhrquist 1976) It has led to the suggestion that also the activit of the renal renin–angiotensin system is under influence of a sodium sensitive cerebra mechanism. That the present IVT infusions, especially of glycerol (causing most pronounce reduction of CSF [Na^+]) induced a rise in PRA before noticeable dehydration had develope appears consistent with that idea.

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Tension and cyclic GMP changes in potassium depolarized rabbit colon muscle

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Abstract

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Potassium depolarization of rabbit colon muscle elicited a contraction consisting of 2 distinct phases, an initial rapid phasic contraction and a tonic contracture. The tonic contraction was, in contrast to the phasic contraction, dependent on the extracellular calcium for its development. There was a correlation between the tension development and the increase of the cyclic GMP level in the K^+-depolarized muscle. Experimental conditions which abolished the tonic contracture, ie glucose omission and treatment with Ca^{++} antagonists (verapamil, SKF 525A) also inhibited the cyclic GMP response. The changes of the cyclic GMP levels were Ca^{++}-dependent. K -ions also changed the cyclic AMP content an effect which was atropine sensitive. From the experimental data obtained in this investigation we suggest that the oscillation of the tension and the cyclic GMP level in the depolarized colon muscle might depend on oscillations in a common intracellular factor probably Ca^{++}

The mechanisms by which various stimulating agents initiate contractile responses in smooth muscles appear to differ Edman and Schild (1961), Briggs (1962) and Hurwitz *et al* (1967) reported that potassium ions initiate a contraction by inducing the influx of extracellular calcium. Acetylcholine, on the other hand seems to influence calcium compartments in the smooth muscle membrane, and it causes a rapid release of calcium from both superficial and sequestering calcium sites in the membrane.

The importance of cyclic nucleotides in regulating Ca -movements in smooth muscle has been discussed by Andersson and Nilsson (1976). The relationship between cyclic nucleotides and contractions elicited by cholinergic drugs has been studied in intestinal smooth muscle (Andersson 1972, Andersson *et al* 1975 Lee *et al.* 1972). It was found that these drugs first reduced cyclic AMP levels, but later increased the content of the cyclic nucleotide. The content of cyclic GMP was constantly raised after stimulation by cholinergic drugs. Potassium ions have been shown to increase the cyclic AMP content of rabbit colon (Andersson 1972) in ductus deferens of the rat KCl also increased the cyclic GMP levels, an effect which was due to the presence of Ca^{++} ions (Schultz *et al* 1973).

In this study the relationship between mechanical effects induced by K^+ ions and effects

[illegible] the cyclic nucleotide levels in colon muscle has been studied. The effect of drugs (vera[illegible]mil, SKF 525A) that influence the transmembrane Ca influx into smooth muscle cells [illegible] potassium induced depolarization has also been investigated.

Methods

[illegible] muscle employed in this study was isolated from rabbit colon as described by Andersson and [illegible]-Lundholm (1970). Segments approximately 2 cm long and 1 cm [illegible]ide were fixed into plastic holders [illegible] suspended in an organ bath containing 30 ml of Krebs buffer solution. The solution [illegible]as bubbled [illegible] O_2–5 [illegible] CO_2 and the temperature of the bath was maintained at 37°C. After 60 min of preincuba[illegible], some of the muscles [illegible]ere immersed in K -rich medium. In the experiments [illegible]here the preparations [illegible] depolarized with K -ions, the composition of the suspension solution in mM was: 124.7 KCl, 2.4 [illegible], 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$ and 11.5 glucose. I[illegible] order t[illegible] deprive the muscles of their [illegible] they were washed repeatedly in calcium-free Krebs solution containing 0.03 mM EGTA, except the last wash where EGTA was omitted from the solution. I[illegible] the experiments where the Ca^{++} an[illegible] (verapamil and SKF 525A) were used the muscles were preincubated with these drugs for 10–15 [illegible] before they were transferred to K -rich medium.

[illegible] preparations used for the determination of the metabolic parameters were pretreated with sotalol [illegible] g/ml) for 10 min, in order to prevent cyclic nucleotide changes due to the liberation of adrenergic [illegible] substances. For the determination of cyclic nucleotides the tissues were frozen in [illegible] medium, [illegible] 12° (Hoechst) containing dry ice. The frozen muscle preparations were homogenized in 2 ml 5 [illegible]. After centrifugation the supernatants were adjusted to pH 7.0, and were then charged on to columns AG1-X8 (200–400, formate form). The cyclic nucleotides were eluted [illegible]ith formic acid. After lyophiliza[illegible] the cyclic AMP content was determined according to Gilman (1970) and cyclic GMP according to [illegible] et al. (1972). The ATP content of the preparations was determined according to Lamprecht & [illegible] (1970).

Results

Effects of K ions on cyclic nucleotide levels

After 60 min of preincubation with normal Krebs buffer solution, the mean concentrations of cAMP and cGMP in rabbit colon were 620 ± 50 and 76 ± 7.0 pmol/g wet weight (Fig. 1), respectively. When the muscles were transferred to a K -rich solution a rapid increase of muscle tension was observed and a significant rise of cyclic GMP was observed within 30 s and 2 min after transferring them to a K -rich buffer (Fig. 1), the content had nearly tripled. Cyclic AMP levels were reduced by about 25% after 30 s ($p < 0.05$), but increases of this nucleotide (Fig. 1) were observed after both 2 min and 5 min periods. In order to exclude the effect of acetylcholine released from cholinergic nerves by K -ions, the effect of K -ions was also investigated on atropinized muscles. In these preparations K -ions still produced a contraction and increased cyclic GMP but the decrease of the cyclic AMP level observed after 30 sec of incubation with K -ions was absent in atropinized muscles (Fig. 2).

Effects of verapamil and SKF 525A on K -induced contraction and nucleotide changes

The potassium-induced contraction in rabbit colon can be divided in a rapid phasic and a [illegible] tonic component (Fig. 3). The main effect of Ca^{++}-antagonists like verapamil and SKF 525A is to block the tonic component of the contraction. In higher concentrations also the phasic contraction is completely blocked (Fig. 3 a, b).

The basal levels of [illegible]

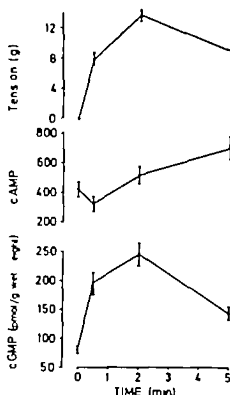


Fig. 1 Influence of K -ions (126 mM) on tension and cy
nucleotide levels in rabbit colon Mean ± S E. (n 10)

pamil or SKF 525A However SKF 525A in a concentration of 1 10^{-4} g/ml complete
blocked both the contraction and the increase of cyclic AMP and cyclic GMP caused b
K -ions (Table I) Verapamil (2 10^{-4} g/ml) reduced the K+-contraction as well as th
potassium induced changes of cyclic nucleotides (Table II). In higher concentrations (10
g/ml) verapamil completely blocked both contraction and nucleotide changes observed afte
incubation in K -rich solution.

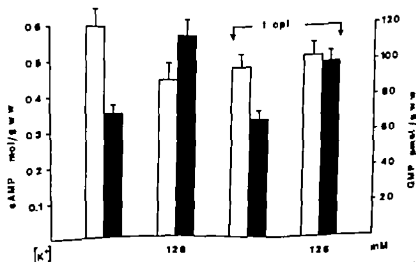


Fig. 2 Cyclic AMP (open bars) and cyclic GMP (filled bars) levels in rabbit colon after incubation
K -high Krebs solution for 30 s in the presence and the absence of atropin 0^{-7} g/ml). Mean ± S

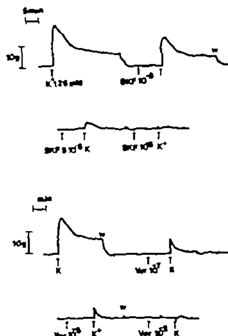


Fig. 1. Effects of different doses (g/ml) of a) SKF 525A and b) verapamil (Ver) on K (126 mM) induced contractions of colon muscle. The muscle was washed with Krebs solution at W

Effects of Ca^{++} omission and re-addition on tension and cyclic nucleotide content

As illustrated in Fig. 3 the mechanical response of colon muscle to K -rich solution consisted of two phases, a rapid phasic and late tonic contraction. When the concentration of calcium was varied in the incubation solution from 0 t 9 mM the appearance of the potassium contraction changed. The amplitude of the phasic component was rather constant when the calcium concentration was varied in the incubation medium (Fig. 4). The tonic contracture disappeared completely when calcium was omitted from the solution. As is depicted in Fig. 4 there was a gradual improvement of tonic contraction when the calcium concentration in the medium increased up to 9 mM.

The omission of Ca^{++} from the physiological buffer solution and the deprivation of Ca^{++} from the muscles with EGTA treatment reduced the cyclic GMP as well as the cyclic AMP

TABLE I Effects of K -ions (126 mM 2 min) and SKF 525A (1 10^{-6} g/ml, 15 min) alone or in combination with each other on tension and cyclic nucleotides in rabbit colon muscles Mean ± S E 6–7 Degree of significance of the difference from control values is noted by * = p 0 05 ** p 0 01

	cAMP pmol/g w w	cGMP pmol/g w w	Tension (g)
Control	340 ± 67	34 ± 5	—
K -ions	490 ± 62*	197 ± 33**	13.9 ± 1**
SKF 525A	400 ± 60	25 ± 7	—
SKF 525A K -ions	310 ± 50	33 ± 4	—

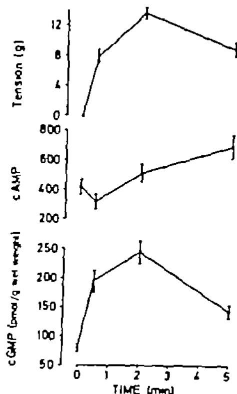


Fig. 1 Influence of K -ions (126 mM) on tension and cyclic nucleotide levels in rabbit colon. Mean ± S.E. (n 10)

pamil or SKF 525A. However SKF 525A in a concentration of 1 10^{-4} g/ml completely blocked both the contraction and the increase of cyclic AMP and cyclic GMP caused by K^+ ions (Table I) Verapamil (2 10^{-6} g/ml) reduced the K^+-contraction as well as the potassium induced changes of cyclic nucleotides (Table II). In higher concentrations (10^{-4} g/ml) verapamil completely blocked both contraction and nucleotide changes observed after incubation in K -rich solution.

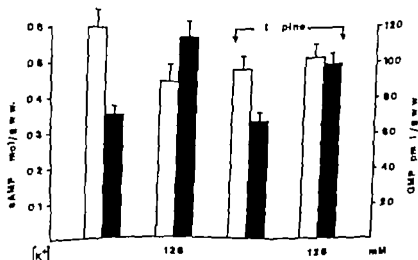


Fig. 2. Cyclic AMP (open bars) and cyclic GMP (filled bars) levels in rabbit colon after incubation in K^+-high Krebs solution for 30 s in the presence and the absence of atropine (8 $10^{-?}$ g/ml). Mean ± S.E. (n = 6).

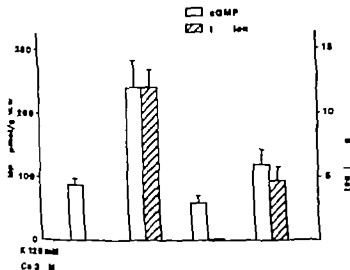


Fig. 5 Influence of Ca^{++} on the effect of K^+ (126 mM) on tension and cyclic GMP levels in colon muscles. Tissue was either preincubated in normal Krebs solution or in Ca^{++}-free medium containing EGTA for 8 min and then transferred for 2 min to fresh medium containing Ca^{++} and K^+ as indicated in the figure. The asterisk indicates readdition of Ca^{++}. Mean ± S.E. (n = 6)

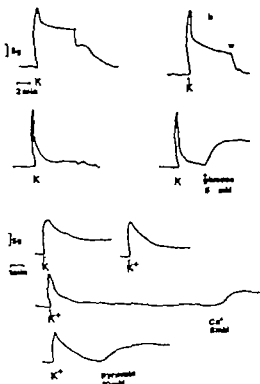


Fig. 6. The effect of removing glucose from the incubation medium on K^+ (126 mM) induced tension contractions (a) and contractions elicited in the presence of glucose. Contractions elicited 20 min (b and f), and 60 min (c) after the removal of glucose. The addition of glucose (5.5 mM, d), Ca^{++} (8 mM, g) and pyruvate (10 mM, h) to muscles incubated in the absence of glucose for 60 min.

TABLE II Influence of different concentrations of verapamil on potassium (126 mM) induced changes of cyclic nucleotide levels and tension Mean ± S E. (n = 6–12). Degree of significance denoted as in Table I

Drugs	Cyclic AMP pmol/g w w	Cyclic GMP pmol/g w w	Tension g
Control	387 ± 60	21.3 ± 2.7	—
K -ions (126 mM)	539 ± 75	121 7 ± 23 4	9.2 ± 1.0*
Verapamil (2 10⁻⁶ g/ml)	373 ± 55	27.3 ± 6 0	—
Verapamil (2 10⁻⁶ g/ml) + K -ions (126 mM)	340 ± 63	95 0 ± 7.8	2.3 ± 0.3
Verapamil (1 10⁻⁶ g/ml)	453 ± 30	1 1 ± 6..	—
Verapamil (1 10⁻⁶ g/ml) + K -ions (126 mM)	468 ± 52	32.0 ± 10.7	0.3 ± 0 1

levels of the muscle. In such muscles, both the contractile and the cyclic GMP elevating effects of K^+ ions were lost. The readdition of Ca (3 mM) to this muscle restored the contractile response to K and also the effects on cyclic GMP and cyclic AMP (Fig. 5).

Effects of glucose omission on tension and cyclic nucleotide content

When glucose was omitted from the incubation medium the tonic contracture induced by K^+ ions gradually disappeared while the phasic contraction was almost unchanged. After 1 h of incubation in a glucose free medium only the phasic contraction could be detected (Fig 6 c) The tonic contracture could be restored either by carbohydrate metabolites (glucose pyruvate) or by a high concentration of Ca (8 mM) (Fig. 6 d, g, h) The increase of cyclic GMP observed in the K -depolarized muscle was abolished in the glucose deficient muscle (Table III). The levels of cyclic AMP and energy-rich phosphate compounds such as ATP and CrP were also lowered. The addition of glucose or pyruvate to the glucose-deficient muscle restored both the tonic contracture and the cyclic GMP level (Fig. 6 and 7). A similar effect was also demonstrated when of CaCl (8 mM) was added (Fig. 6 and 7). As is evident from Figure 8 an increase of tension in the glucose-deficient K -depolarized muscle is associated with an increase of cyclic GMP This covariation occurred both with carbohydrates and Ca^{++}-ions.

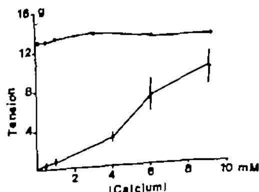


Fig. 4 Effects of different concentrations of calcium in incubation solution on potassium induced contraction of colon muscle. Dotted area amplitude of phasic contraction. Solid line tonic contraction. Mean ± S E. (= 8)

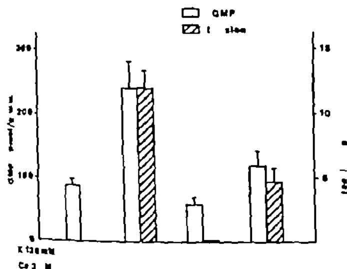


Fig. 3 Influence of Ca^{++} on the effect of K (126 mM) on tension and cyclic GMP levels in colon muscles. ... were either preincubated in normal Krebs solution or in Ca^{++}-free medium containing EGTA for ... and then transferred for 2 min to fresh medium containing Ca^{++} and K as indicated in the figure ... asterisk indicates readdition of Ca^{++}. Mean ± S.E. (n = 6).

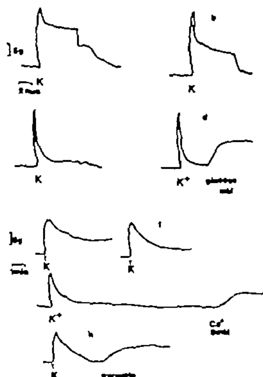


Fig. 4. The effect of removing glucose from ... incubation medium on K (126 mM) in... and sustained contractions, and contrac... ... elicited in the presence of glucose. Con... ... elicited 20 min (b and f), and 60 min ... after the removal of glucose. The addition of glucose (5.5 mM, d), Ca^{++} (3 mM, g) and ... [illegible] (10 mM, h) to muscles incubated in ... absence of glucose for ...

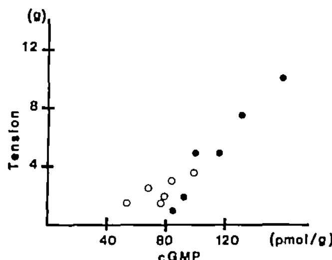


Fig. 7 Effects of Ca++ (3 mM open circles) and glucose (5.5 mM closed circles) on the tension and cyclic GMP levels in colon muscles suspended in a glucose free Krebs solution for 1 h.

Discussion

The mechanical response of rabbit colon muscle to K -rich Krebs solution consisted of two distinct phases an initial rapid rise of tension the phasic response, and an ensuing tonic contraction A similar effect of high potassium medium has earlier been shown by Durbin and Jenkinsson (1961), Urakawa and Holland (1964) and Imai and Takeda (1967) on guinea pig taenia coli and by Somlyo and Somlyo (1969) on rabbit mesenteric vein. The tonic component of the contraction seemed to be most sensitive to drugs such as Verapamil and SKF 525A (Fig. 3) Higher concentrations of these drugs also affected the phasic response Verapamil and SKF 525A have been shown to block transmembranal Ca influx in smooth muscles (Fleckenstein *et al* 1969 Haeusler 1972, Kalsner *et al* 1971) Briggs (1962) and Hinke *et al* (1964) suggested that the K contraction of arterial smooth muscle depends on an influx of Ca + from the extracellular space. According to our experiments we suggest that this interpretation may also apply to the tonic contraction of colon muscle. The degree of tonic contracture was closely related to the extracellular calcium concentration (Fig. 4) while the phasic contraction was almost independent of extracellular calcium in the depolarizing solution.

The present data show that the influx of Ca++ is also a very important factor for the regul

TABLE III Effects of glucose omission on cyclic nucleotides ATP and CrP levels in polarized and K depolarized colon muscle. The metabolites were determined after an incubation period of 60 min in K -rich Krebs buffer and 2 minutes after the glucose-addition. Mean ± S.E. n 6 Degree of significance denoted as I Table I

Solutions + additions	Cyclic AMP pmol/g w w	Cyclic GMP pmol/g w w	ATP mol/g w w	CrP nmol/g w w
Normal Krebs	271 ± 42	70 ± 7.0	590 ± 90	299 ± 67
K rich Krebs	300 ± 50	133 ± 15	555 ± 50	197 ± 51
K+ rich Krebs without glucose	170 ± 10	88 ± 2	387 ± 9	72 ± 7
K -rich glucose + 5.5 mM glucose	210 ± 20	106 ± 7	—	—

...on of cyclic GMP levels in colon muscle. The increase of cyclic GMP observed during ...e K⁺-contraction seemed to be Ca^{++}-dependent, as it was absent in Ca-depleted muscles (Fig. 5) and in muscles where the transmembrane influx of Ca^{++} was blocked by drugs (Table I; Fig. 4). In ductus deferens and submaxillary glands Schultz *et al.* (1973) also found Ca^{++}-dependent increase of cyclic GMP levels by cholinergic agonists and by KCl. These authors suggested that the most probable reason for the cyclic GMP increase was a stimulation of the guanylate cyclase activity by Ca^{++}. Hardman *et al.* (1971) have shown that Ca^{++} is capable of stimulating guanylate cyclase obtained from various tissues. In another study we have also demonstrated an activation of colon muscle soluble guanylate cyclase by high concentrations of Ca^{++} (Nilsson and Andersson 1977).

The transient decrease of cyclic AMP following a K⁺-contraction could possibly depend on release of the cholinergic transmitter since this effect was blocked by atropine. Moreover, cholinergic drugs have been shown to produce a transient decrease of cyclic AMP levels in rabbit colon (Andersson 1973). Paton and Aboo Zar (1968) showed that K⁺-ions release acetylcholine from intestinal muscle preparations.

The observations that β-adrenoceptor mediated relaxation are accompanied by elevated cyclic AMP levels (Triner *et al.* 1971, Andersson 1972) and cyclic GMP levels are elevated by contracting agents (acetylcholine, KCl, Lee *et al.* 1972, Schultz *et al.* 1973, Andersson *et al.* 1975) may support a regulatory role of cyclic nucleotides in the contraction-relaxation cycle of smooth muscle. There has often been confusion and controversy as to the relative importance of the cyclic nucleotides in smooth muscles, but cyclic AMP seems to promote relaxation in most smooth muscle and the contraction is mostly associated with increased cyclic GMP levels. It has also been found that cyclic AMP stimulated Ca-binding to smooth muscle microsomes is antagonized by cyclic GMP (Nilsson and Andersson 1977).

Any direct role for cyclic nucleotides in the K⁺-induced contraction seems improbable. The most probable mechanism seems to be that the depolarization caused by K⁺-ions induced membrane changes in such a way as to permit the entrance of calcium into the cytoplasm. This has been suggested by Hinke *et al.* (1964) and Hurwitz and Suria (1971). However, the increase of cyclic GMP observed during K⁺-contraction might have a modulating effect on contraction. It could possibly promote maintenance of the tonic contraction. Under conditions which suppress cyclic GMP levels viz. glucose omission or calcium antagonism (Fig. 7; Table I–III) only phasic contraction was elicited. The tonic contracture and the increase of cyclic GMP levels in the glucose-free muscles was restored by carbohydrates. This fact may indicate that glucose or pyruvate are immediately utilized in the development of contracture by depolarized muscles an observation which is in accordance with the results of Axelsson *et al.* (1965). An increased tonic contraction in the glucose-free muscle can, however, be obtained by increasing the Ca^{++}-concentration in the extracellular medium (Fig. 6). This indicates that the energy supply to the contractile elements is not a limiting factor. The cytoplasmic Ca^{++}-concentration might instead have been too low in the glucose-free muscle in order to activate the contractile proteins and to increase the cyclic GMP content. It is, however, not possible from this study to determine if in the K⁺-depolarized colon muscle, the cyclic GMP and the tension changes are causally related, or if the covariation is dependent on a common intracellular messenger such as Ca^{++}.

We are indebted to Mrs Ingrid Salomonsson, Mrs Eva Hägglund and Mr Sverker Börjeson for assistance. Financial support has been provided by the Swedish State Medical Research Council (04 and Magnus Bergvalls Foundation.

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blood. Liver function was estimated from the hepatic oxygen uptake, the hepatic clearance of Indocyanine Green (ICG) and the hepatic elimination rate of ethanol. The operational technique, flow measurements, and analytical procedures and calculations have all been described elsewhere in detail (… 1973, Kramp, Larsen and Munck 1976). Secretin (pure, porcine secretin, G.I.H. Research Unit, … Department, Karolinska Institutet, Stockholm) was given i.v. in isotonic NaCl solution, pH 3. Indomethacin (Merck, Sharp & Dohme B. V. Haarlem, Netherlands) was dissolved in 90 % ethanol and diluted to 15 ml 0.9 % sodium chloride solution containing 150 mg sodium bicarbonate and heated to …°C. The results are given as mean values ± S.E. and the effects of secretin and Indomethacin tested by the Student's test for paired comparisons.

Results

The results from experiments on 8 cats in which secretin was infused at a rate of 2 U kg^{-1} h^{-1} are listed in Table I and II. Infusion of secretin was always followed by an immediate rise in bile flow which stabilized in the second or third 15-min period and remained stable for the rest of the experimental period of up to 2 h. A barely significant rise in erythritol clearance was also noticed. Thus the bile flow which initially was lower than the erythritol clearance finally exceeded it. It appears therefore that the increase in bile flow was caused almost exclusively by a change in the ductular net fluid transport, which reversed from a net reabsorption to a net secretion of fluid. The increase in flow was accompanied by an increase in the biliary excretion rate of Na^+, K^+, Cl^- and HCO_3^- whereas no change was observed in the excretion rate of bile acids. In 5 cats where liver hemodynamics and liver metabolism were examined, secretin did not affect hepatic arterial blood flow (12.6 ± 2.0 ml kg^{-1} min^{-1}), portal venous blood flow (21.8 ± 3.9 ml kg^{-1} min^{-1}), hepatic arterial conductance (0.09 ± 0.01 ml kg^{-1} min^{-1} mmHg^{-1}), portal conductance (3.5 ± 0.3 ml kg^{-1} min^{-1} mmHg^{-1}) or mesenteric conductance (0.17 ± 0.03 ml kg^{-1} min^{-1} mmHg^{-1}). Neither was any change observed in the hepatic oxygen uptake (45.0 ± 6.5 µmol kg^{-1} min^{-1}), ICG-clearance (4.9 ± 1.4 ml kg^{-1} min^{-1}) or hepatic ethanol elimination (29.0 ± 5.1 µmol kg^{-1} min^{-1}).

In 8 cats secretin was infused at a rate of 2 U kg^{-1} h^{-1} as above. Once bile flow was stable, Indomethacin was given i.v. as a single dose of 25 mg kg^{-1}. Results from these experiments are listed in Table I and II. Indomethacin reduced bile flow significantly but this reduction was mainly due to a reduction in canalicular bile production. There was also a slight reduc-

Table I. Effects of secretin and of secretin + Indomethacin on bile flow and erythritol clearance. Mean values ± S.E. I: 8 expts. before (A) and during (B) infusion of secretin 2 U kg^{-1} h^{-1}. II: 8 expts. during (C) infusion of secretin 2 U kg^{-1} h^{-1} and (D) supplemented with Indomethacin 25 mg kg^{-1}

	I		II	
	A	B	C	D
Bile flow (µl kg^{-1} min^{-1})	11.4 ± 0.7	21.1 ± 0.8***	22.7 ± 2.0	17.8 ± 1.9*
Bile-to-plasma ratio of Erythritol	1.45 ± 0.08	0.85 ± 0.04***	0.86 ± 0.06	0.91 ± 0.08
Erythritol clearance (µl kg^{-1} min^{-1})	16.3 ± 0.8	17.8 ± 0.6	19.2 ± 1.6	15.8 ± 1.4**
Ductular net fluid transport (µl kg^{-1} min^{-1})	− 4.8 ± 0.8	+ 3.3 ± 0.9***	+ 3.5 ± 1.5	+ 2.0 + 1.6

* 0.05 > p > 0.01, ** 0.005 < p < 0.010, *** p < 0.001

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The effect of secretin on bile production, splanchnohepatic hemodynamics and liver function in cats

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Abstract

LARSEN J A and N KRARUP *The effect of secretin on bile production, splanchnohepa hemodynamics and liver function in cats* Acta physiol scand. 1978 *102* 420–424

The effect of secretin on bile flow biliary clearance of ¹⁴C-erythritol and bile composition as studied fasting, chloralose anesthetized cats. Secretin (2 U kg⁻ h⁻) increased bile flow by 85% and increased rates of excretion of Na Cl⁻ and HCO Bile flow increased from alues below t values above the er thri clearance, which did not change significantly It is therefore concluded that secretin altered ductular trans of fluid from a net reabsorpt on to a net secretion of bicarbonate rich fluid. Secretin did not affect splanchno-hepatic hemodynamics or the overall function of the liver and Indomethacin did not sig ficantly alter the response t secretin

The choleretic effect of secretin is well established in humans as well as in a variety of anim (Erlinger and Dhumeaux 1974) but only few reports deal with the choleretic action secretin in cats (Tanturi, Ivy and Greengard 1937 Scratcherd 1965) It was recently report (Krarup Larsen and Munck 1976) that infusion of prostaglandin E and E_2 influenced bi flow in cats in a way which mimicked the effect of secretin in other animals. The pres experiments were therefore undertaken with the purpose of examining the choleretic actio of secretin in cats together with the possible role of prostaglandin in secretin choleresis. Al the effect of secretin on splanchnic hemodynamic parameters and liver metabolism w followed.

Methods

Fasting cats (weight 3.4 ± 0.2 (S.E.) kg) anesthetized with chloralose (50 mg·kg⁻¹) and Nembutal (30 m given i.p. were used fo the experiments. Catheters were placed i the femoral rtery and ein, the por vein and a hepatic vein for blood sampling, infusion and pressure measurements. Flowprobes were plac around the portal vein and the hepatic artery and blood flow was measured by an electromagnetic flo meter (Nycotron, Oslo). Bile was collected from a catheter placed in the choledochus after ligation of c cystic duct. To compensate for the abrupted enterohepatic circulation of bile acids, taurocholate (0.2 µmo kg⁻¹·min⁻¹) was infused continuously The canalicular bile flow was estimated by the biliary clearance ¹⁴C-erythritol and the net reabsorption/secretion of fluid in the bil ductules was calculated as the differen between erythritol clearance and bile flow The biliary excretion rate of bile acids and inorganic ions

… secretin significantly and the results do not therefore support the assumption that the effect of secretin on bile ductuli is mediated via prostaglandins.

In our experiments secretin was found to be without effect upon the sphlanchno-hepatic vascular bed; there are only a few and conflicting reports to be compared with these results. … (1970) found that close intraarterial injections resulted in an increase in flow and conductance of the mesenteric arteries in cats, whereas injection of secretin into the hepatic artery was followed by an indirect and delayed decrease in flow and conductance. In contrast to these findings Richardson and Withrington (1976) found that secretin increased conductance and flow in the perfused arterial vascular bed of the dog's liver. The discrepancies between the findings may be due to differences in species, dosage and experimental technique.

In conclusion the present results indicate that the biliary effect of secretin in the cat is mainly if not exclusively localized on the bile ductular epithelium and is not accompanied by or secondary to hemodynamic changes or changes in the overall function of the liver.

The skilled technical assistance of Miss Karin Donby is gratefully acknowledged.

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TABLE II Effects of secretin and of secretin + Indomethacin on the rates of excretion of various ponents. The rates of biliary excretion of anions and cations are given as μmol kg^{-1} min values ± S.E. I 8 expts. before (A) and during (B) infusion of secretin 2 U kg^{-1} h^{-1} II. during (C) infusion of secretin 2 U kg^{-1} h^{-1} and (D) supplemented with Indomethacin 25

	I		II	
	A	B	C	D
Na	1 74 ± 0.09	3.18 ± 0.13	3 42 ± 0.35	2.76 ± 0.30***
K	0.05 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0 08 ± 0 01**
Cl^-	1.25 ± 0.11	2.26 ± 0.11	2.44 ± 0.22	1.87 ± 0.19**
HCO_3^-	0.32 ± 0.02	0.82 ± 0.05	0.83 ± 0.14	0.63 ± 0.10*
Bile acids	0.29 ± 0 01	0.31 ± 0 01***	0.36 ± 0 01	0.31 ± 0.03***

$0.01 < 0 < 0.02$, $0.001 < p < 0.005$ $p < 0.001$

tion in the net ductular fluid secretion rate and the rates of excretion of electrolytes change in the excretion rate of bile acids. In a few experiments Indomethacin was admin prior to the infusion of secretin the responses to secretin were essentially the same as obtained when no pre treatment was given.

Discussion

The observed effects of secretin on bile flow and composition are similar to those fo conscious dogs given the same amount of secretin (Preisig, Cooper and Wheeler 1962, and Grossman 1969 Jones, Geist and Hall 1971 Soloway *et al* 1972, Russell, Sear Jones 1975 and Kaminski, Ruwart and Jellinek 1975) This indicates that the anes applied is without major importance for the choleretic effect of secretin. The marked c in ductular net fluid transport the lack of effect on bile acid secretion as well as the inc in the excretion rate of bicarbonate are all in accordance with the present view on the eff secretin on bile production. According to this, the main effect of secretin is the secretion bicarbonate-rich fluid in bile ductuli (Boyer and Bloomer 1974, Strasberg *et al* 1975). I present experiments secretin changed a net fluid reabsorption in the ductuli to a net secre Correspondingly the bile-to-plasma ratio of erythritol changed from above unity (1 4 below (0 85). A ratio greater than 1.0 was also found during control conditions in (Wheeler Ross and Bradley 1968) and rabbits (Erlinger *et al* 1970) whereas a ratio sm than 1 0 was found in guinea pigs (Forker 1967) and dogs (Erlinger and Dumont 197 well as in humans (Prandi *et al* 1975) Boyer (1971) found a value close to 1.0 in the perf rat liver These discrepancies may be explained by differences in the nutritive state of experimental animals and thus the secretion rate of secretin. This explanation is suppo by the experiments of Strasberg *et al* (1975) on monkeys in which bile-to-plasma rati erythritol was greater than 1 0 during fasting and smaller than 1.0 after feeding.

The results demonstrate that the effects of secretin on bile production in the cat similar to those obtained with prostaglandins (Krarup, Munck and Larsen 1976). Howe Indomethacin, which inhibits the synthesis of prostaglandins (Vane 1971 Smith and La 1971 Afonso *et al* 1974 and Clark and Cumby 1975) did not aff tular respo

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Effect of the nonionic detergent Triton X 100 on sodium permeability of the myelinated nerve fibre of *Xenopus laevis*

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Abstract

Brismar, T. and B. Rydqvist. *Effect of the nonionic detergent Triton X 100 on sodium permeability of the myelinated nerve fibre of* Xenopus laevis. Acta physiol. scand. 1978. 102. 425–433.

The effect of the nonionic detergent Triton X-100 (TX 100) on the Na permeability (P_{Na}) properties of the nodal membrane in myelinated nerve fibres of *Xenopus laevis* was analysed with potential clamp technique. Application of TX-100 caused a rapid initial decrease in P_{Na} that was reversible at wash out as well as an irreversible block. Both effects depended on [TX 100] and duration of exposure. The reversible reduction of P_{Na} at the steady state was 50 % at 40–60 µM TX 100. The slope of the Hill plot for the reaction was 1.1 indicating deviation from a first order reaction. The equilibrium dissociation constant (K_D) for ... was 6.4 × 10^{-5} (M). K_D calculated from the rate constants for onset and offset of the reversible reaction (K_D = k_2/k_1) was 1.3 × 10^{-4} (M). The possibility that the action of TX-100 involves membrane ... is discussed.

The conduction of impulses in nerve is associated with specific changes in the ionic permeability properties of the membrane (*cf* Hodgkin 1964). The potential and time relations of the permeability mechanisms have been described for squid giant axons (Hodgkin and Huxley 1952) and myelinated nerve fibres (Frankenhaeuser and Huxley 1964). The structure of the permeability sites is still unknown. Some evidence indicate that proteins constitute part of it, *cf* Armstrong, Bezanilla and Rojas (1973) have found that the proteolytic action of pronase on the inside of squid axons selectively destroys the mechanism for Na permeability inactivation. Several investigators have tried to characterize chemically the permeability sites on the basis of experiments with substances which affect the permeability properties and have more or less well defined action sites (*cf* Narahashi 1974).

It has been found that nonionic detergents block the conduction in nerve and the neuromuscular transmission (Skens and Soehring 1952, Soehring, Dass and Stave 1952, Zipf and Dittmann 1964, Dittmann 1973). The nonionic detergents may interact with biological membranes in several ways. A perturbation of the lipid phase may alter the physical properties of the bilayer. At high concentrations nonionic detergents possibly form mixed

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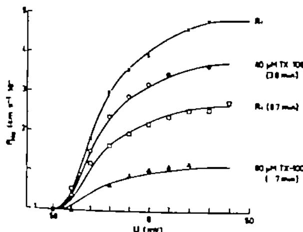


Fig. 1. Effect of TX-100 on peak Na permeability (P_{Na}) vs. potential (U) curve. Membrane polarized to [illegible] mV between test pulses. P_{Na} calculated from records of membrane current obtained in different external solutions applied in the following order: Ringer solution (X), after 3.0 min in 40 µM TX 100 (●), after [illegible] min in 80 µM TX 100 (Δ), and after 8.7 min in Ringer's solution (□). Axon 1. Temp. 11–13°C.

tion (Frankenhaeuser 1960, Brismar 1977 b). Experiments with conditioning pulses of various amplitudes indicated that TX-100 did not affect the steady-state relation between P_{Na} inactivation and membrane potential.

When high concentrations of TX 100 (160 µM in the present experiments) were applied or when TX-100 in lower concentrations acted for long durations (>30 min) then suddenly a rapid increase in membrane leak current appeared which terminated the experiment. Still lower concentrations TX 100 did not affect the leak and the capacitive current amplitude and did not cause an increased rate of Na entry which generally was associated with the 'run down' of the isolated fibre. The rate of Na entry was monitored by repeated measurements of the Na equilibrium potential. The time course of the action of TX-100 on the peak P_{Na} was calculated from measurements of the change in amplitude of the peak Na current associated with a positive potential pulse applied repeatedly. Fig. 2 shows the time course of the change in peak P_{Na} calculated in this way. The size of the effect was clearly related to the concentration of TX-100 in the external solution and the duration of the exposure. A steady state was not reached but the initial phase of decrease in P_{Na} was succeeded by a slow continuous depression. This together with the observed persistence of the reduction in P_{Na} after wash out of TX-100 indicated that TX-100 had a partially irreversible action.

An hypothetical model of TX 100 action

In order to account for the finding that TX-100 had both reversible and an irreversible effect on P_{Na} and to provide a basis for a quantitative description it was necessary to put up a hypothetical model for the reaction between TX-100 molecules and acceptor sites on the nodal membrane. It was assumed that the reversible effect was due to a reversible binding of TX-100 molecules on acceptor sites on the nodal membrane, thereby blocking the Na per

micelles with different phospholipids (Dennis 1974). Recent studies have demonstrated that nonionic detergents can bind to proteins and in particular to more hydrophobic proteins (Mikano Reynolds and Tanford 1973 Simons, Helenius and Garoff 1973 Helenius and Simons 1975) The proteins investigated have been shown to have both high and low affinity sites.

In the present study the action of a nonionic detergent, Triton X 100 (TX 100), on the Na permeability mechanism in myelinated nerve fibres was investigated. Potential clamp experiments were performed on the node of Ranvier in single fibres dissected from the frog (*Xenopus laevis*) TX 100 decreased the Na permeability The magnitude of its blocking action increased with the concentration and the time of application. The effect of TX 100 was only partially reversible. The results are tentatively described in terms of agon receptor kinetics.

Methods

Myelinated nerve fibres were dissected from the sciatic nerve of the frog (*Xenopus laevis*). Single fibres w isolated and mounted in a perspex recording chamber The potential clamp technique was similar to the c earlier described by Dodge and Frankenhaeuser (1958) with minor modifications (Brismar and Frank haeuser 1972). Some of the experiments were made in a recording chamber allowing very fast solut changes. The half time for the effective solution change at the nodal membrane was 100–150 ms for [N changes (Brismar 1977). The diffusion of TX 100 into the nodal gap may however be consideral slower than for Na.

TX 100 was purchased from Rohm and Haas, Sweden, and used without further purification. Tl detergent belongs to series of homologes with the general formula $C_{m}H_{2m+1}$–$C_6H_4O(CH_2CH_2O)_nH$. T mean molecular weight of TX 100 is 628 (Handbook of Physical Properties, Rohm and Haas Surfacta CS-01a Triton surfaceactive agents, The nonionic octylphenoxiethanols (OPE) series, Cs-401/cf). F TX 100 m is 8 and the mean number of ethyleneoxide groups, n, is 9.8. The commercially available dete gent is not homogeneous but a mixture of detergents with a Poisson distribution of n (Sachat and Gree wald 1967).

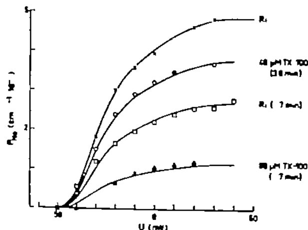
Test solutions were made with various concentrations of TX 100 (40, 60, 80 and 160 µM) The electroly composition was in all test solutions 112 mM NaCl, 2.5 mM KCl, 2.0 mM $CaCl_2$ and 2.5 mM Trizma buffer pH 7 4 (25°C).

Nomenclature. The membrane potential (U) is inside potential minus outside potential. Outward curre are consequently positive.

Results

Effects of TX 100 on Na permeability

The effect of the detergent TX 100 on the Na permeability (P_{Na}) in single myelinated nerv fibres was analysed with potential clamp technique The membrane currents associated with positive potential pulses were recorded. During exposure to TX 100 the amplitude of the Na current decreased. A slight decrease in the delayed outward potassium current (I_K) was also observed. This latter effect was not studied further The peak P_N was calculated from the peak Na current and the constant field equation (Dodge and Frankenhaeuser 1959). Fig. 1 shows peak P_N *vs.* membrane potential (*U*) before, during and after successive application of 40 and 80 µM TX 100 in Ringer's solution. A smooth curve was fitted to the symbols representing P_{Na} before TX 100 was applied, and from this curve the other curves were obtained by scaling the ordinates with constant factors. It is evident that TX 100 decreased the available P_{Na} but did not affect the potential dependence of P_{Na} activation. The membrane was negatively polarized to −120 mV between the test pulses, which removed P_{Na} inactiva-



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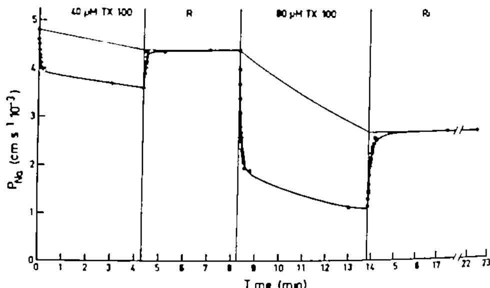
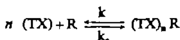


Fig. 2. Time course of TX 100 action on peak P_{Na}. P_{N} calculated from records of membrane curr associated with test pulses to U = 0 mV repeated 1 per s, and from complete potential clamp runs (Fig Thin line represents hypothetical time course of irreversible decrease in P_{Na}. Axon 1. Temp. 11–13°C.

meability sites. In a reversible reaction n molecules of TX 100 (TX) react simultaneously wi some acceptor (*e.g.* a receptor) to produce the effect



$$n\ (TX) + R \underset{k_1}{\overset{k}{\rightleftharpoons}} (TX)_n R$$

The ratio $[(TX)_n R]/[R + (TX)_n R]$ is the fractional occupancy due to the reversible reacti of receptor sites which is denoted y_t^{rev} at time t and y_{∞}^{rev} at steady state.

The change in y_t^{rev} during onset and offset should thus satisfy the classical principles f receptor kinetics (Waud 1968). Accordingly the rate constants (k and k_1) were obtained fro the experimental time constants for onset and offset and the dissociation constant, K_D(= k k_1), from the steady state curve.

An irreversible decrease in P_N was associated with the exposure to TX 100. The irreversib effect of TX 100 was separated from the reversible changes by measuring P_{Na} at steady sta after wash out of the TX 100 solution and interpolating between this P_{Na} value and the val prior to the exposure of TX 100. For the interpolation the irreversible changes were treat as if they followed an exponential time course with a complete irreversible block of P_{Na} infinite time. The reversible occupancy (y_t^{rev}) was calculated from

$$y_t^{rev} = \frac{P_{Na_0} - P_{Na_t} - \Delta P_{Na_t}^{irrev}}{P_{Na_0} - \Delta P_{Na_t}^{irrev}}$$

where P_{Na_0} and P_{Na_t} is P_N at beginning and time t of exposure to TX 100 and $\Delta P_{Na_t}^{irrev}$ is th interpolated size of the irreversible decrease in P_{Na} at time t.

Kinetics of TX 100 action on Na permeability

The time course of the effect of various concentrations of TX 100 on the peak P_{Na} was plotte as shown in Fig. 2 and the reversible fractional occupancies (y_t^{rev}) were

TABLE I. Irreversible effect of TX-100 on P_{Na}

[TX 100] (μM)	y_∞^{rev}	$n=1$: $\frac{1-y_\infty^{rev}}{y_\infty^{rev}}$ [TX 100] (M 10^{-6})	$n=1.75$: $\frac{1-y_\infty^{rev}}{y_\infty^{rev}}$ [TX 100]$^{1.75}$ (M$^{1.75}$ 10)
40	0.19	170	1.42
40	0.12	287	2.44
40	0.30	93	0.77
60	0 [illegible]	27	0.24
60	0.71	4	0.29
80	0.58	58	0.78
80	0.66	41	0.56
160	0.71	65	1.44
		96 ±91	0.91 ±0.72

eqn. 2. The thin line drawn in Fig. 2 represents the exponential decrease of P_{Na} due to the irreversible reaction. It is evident that, according to the analytical model presently used, the reversible process was at steady state prior to wash out of TX 100 solutions. Therefore y^{rev} at the end of the exposure to 40 μM and 80 μM TX 100 in this experiment represents y^{rev} at equilibrium (y_∞^{rev}) at these concentrations of TX-100. Values of y_∞^{rev} from this and similar experiments are listed in Table I. The corresponding values of the equilibrium dissociation constant (K_D) were calculated with $n=1$ and $n=1.75$ respectively. A Hill plot of $y_\infty^{rev}/(1-y_\infty^{rev})$ vs. [TX 100] had a slope of 1.75 (correlation coeff. $=0.40$) meaning that a reaction model in which two TX 100 molecules bind to one receptor better describes the present data than a one-to-one reaction. Other, more complex, interpretations will be described below.

The time course for the reversible change in peak P_{Na} during onset of 40 μM TX-100 and during offset is plotted in Fig. 3. The reversible fractional occupancy y^{rev} was calculated according to eqn. 2 and the time constants were calculated from semilog plots as shown in the Figure (τ_{on} 3.7 s and $\tau_{off}=4.1$ s). Similar calculations were made of the time constants related to 80 μM TX-100 which gave $\tau_{on}=5.1$ s and $\tau_{off}=8.1$ s. The rate constants k and k were calculated from the time constants. Assuming $n=1.75$ as obtained from the Hill plot reasonable agreement between k in 40 and 80 μM was present (1.37 10^{4} and 1.06 10^{4} s^{-1} M$^{-1.75}$ respectively). Assuming $n=1$ or $n=2$ gave a larger difference between k in the two concentrations. k_2 (and τ_{off}) varied much in these measurements (mean value 0.18 s^{-1}). This was also the case for K_D. K_D calculated as k_2/k_1, using $n=1.75$ and the mean values of k and k for 40 and 80 μM was 1.5 10^{-} M$^{1.75}$. This value is of the same order of magnitude as the value found in the steady-state analysis (0.91 10^{-} M$^{1.75}$). If the irreversible component of the decrease in P_{Na} is assumed to follow an exponential time course (indicated by the thin line in Fig. 2) it is possible to calculate the time constant τ_{ir} and hence the rate constant (k_3) for this process. In Fig. 4 $1/\tau_{ir}$ versus [TX 100] is plotted for the same fibres as used in the experiments for the steady-state analysis of the reversible reaction. If a first order reaction is

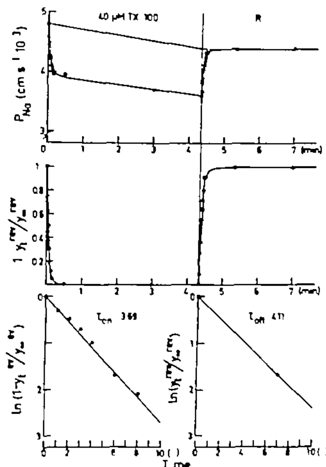


Fig. 3. Time course of change in peak P_{Na} during onset of 40 µM TX 100 and during offset (*top*). Time constants of reversible action of TX 100 (τ_{on} and τ_{off}) obtained by calculation of reversible occupancy (y^{rev} eqn. 2), calculation of $(1-y_t^{rev}/y_\infty^{rev})$ (*middle*), and the natural logarithm (plotted on different time scale, *bottom*). Straight lines (*bottom*) solutions of eqns. 2 and 3 for indicated values of exponential time constants. Axon 1 Temp. 11 13°C.

assumed the slope of a regression line fitted to these points should give a value of k_i according to the eqn. $1/\tau_{ir} = k$ [TX 100]. However the points do not fit a straight line well, *i.e.* the irreversible reaction may deviate from a first order reaction.

Discussion

A large number of chemicals block the specific cation permeability of excitable membranes (*cf* Narahashi 1974). Reagents with defined action sites on proteins, *e.g.* p-chloromercuribenzoic acids (PCMB and PCMBS) and nitrobenzenes block the nervous action potential

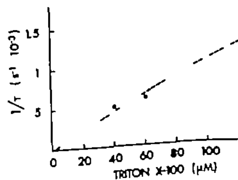


Fig. 4. Dependence of rate of irreversible block of P_{Na} on [TX 100]. Exponential time constant (τ_{ir}) calculated from measurements of irreversible change in P_{Na} after immersion in TX 100 solutions. Necessary assumptions about irreversible reaction described in text. Rate constant (k_i) defined by eqn. $1/\tau_{ir} = k_i$[TX 100] was 11.0 ± 5.0 (s^{-1} M^{-1}). Interrupted line solution of this eqn. for $k_i = 11.0$. Temp. 11 13°C.

through a suppression of P_{Na} and P (Smith 1958, Cooke *et al.* 1968). Several of these compounds have an irreversible action.

The present investigation shows that application of TX 100 decreases P_{Na} in the nodal membrane of frog myelinated nerve fibres. When solutions of low concentrations of TX 100 (40 μM and 80 μM) were applied for short times the decrease in P_{Na} was to its main part reversible at wash out of the TX 100 solution. A large irreversible change on the Na permeability mechanism appeared at high [TX 100] or when long exposure times were tried. At such conditions there was finally a rapid increase in membrane leak conductance. The present findings are in agreement with earlier observations that nonionic detergents block the impulse conduction in nerve and the neuromuscular transmission (see Introduction). It has similarly been shown that TX 100 affects excitability in frog muscle fibres, thus Rydqvist (1977) found that it decreased the maximum rate of rise of the conducted action potential.

With the potential clamp technique used in the present experiments it was possible to monitor the time course of TX 100 action on the nodal membrane by recording the membrane currents associated with repeated potential pulses of a constant amplitude. This, in combination with the short time for solution change (half time 100–150 ms), gave a satisfactory resolution for an analysis of the kinetics of TX-100 action. The analysis was, however, complicated by the irreversible decrease in P_{Na}, which accompanied the application of TX-100 solutions. The size of the irreversible component of TX-100 action during onset could not be directly measured, but was appreciated by interpolation between P_{Na} prior to the application of the test solution and P_{Na} at steady state after washout. The analysis of the reversible reaction strongly suggested a deviation from a first order reaction between TX-100 molecules and membrane receptors. From the Hill plot *n* was estimated to be 1 75 which gave a dissociation constant K_D 0.91 10− $M^{1.75}$ Similarly Rydqvist (1977) found that *n* = 1.57 best fitted the measurements of TX 100 action on the maximum rate of rise of the action potential in frog muscle. In general, many pharmacological effects on excitable membranes do not follow first order reactions (Rang 1971) and often give Hill coefficients greater than one. If *n* is an integer this can be interpreted as described in eqn. (1) *i.e.* *n* ligands react simultaneously with a receptor to give the effect. An alternative interpretation is that the measured effect, in this case the change in P_{Na}, cannot be translated to the formation of the reaction product. It is for example possible that several receptors are connected to one Na channel. This assumption is not entirely hypothetical since there are reasons to believe that the opening of a Na channel is dependent on the cooperation of several (in squid axons three) gating molecules (Hodgkin and Huxley 1952, Keynes and Rojas 1974) which might correspond to the reaction sites of the TX 100 molecules. The finding that *n* is a noninteger could be accounted for by assuming an allosteric effect, *e.g.* the affinity of sites are mutually influenced by the occupation of other sites (Monod, Wyman and Changeux 1965).

Nonionic detergents can interact with the lipid bilayer phospholipids and hydrophobic proteins. It is likely that the permeability blocking action is due to a reaction with membrane proteins associated to the permeability mechanism. TX 100 has been used for the solvation of the acetylcholine receptor glycoprotein from different electrical organs (Miledi and Potter 1971, Meunier, Olsen and Changeux 1972). The irreversible decrease in P_{Na} was considerably slower than the reversible effect of TX-100, and an increase in leak conductance appeared

when a large part of the Na permeability mechanism had been irreversibly damaged. The mechanism for the irreversible effects might similarly be a solvation of the proteins through the lipid-detergent interaction from the permeability sites of the nodal membrane, thus causing a permanent decrease in P_{Na} succeeded by an increase in the leak conductance.

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Dose-dependent inhibition of sensory nerve activity in the feline dental pulp by anti-inflammatory drugs

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Abstract

AHLBERG, K. F. *Dose-dependent inhibition of sensory nerve activity in the feline dental pulp by anti-inflammatory drugs.* Acta physiol. scand. 1978. *102.* 434–440.

The aim of the present study was to investigate whether substances known to antagonize algogenic agents can block an experimentally induced increased sensory nerve activity in the dental pulp of the cat. Increased neural activity following repeated brief heat stimulation has been shown to be characteristic of the individual cat and can be utilized for intraindividual comparative studies on such increased activity. When indomethacin, diclofenac sodium or naproxen were given intravenously as pretreatment prior to stimulation, a consistent blocking effect on expected impulse activity as compared to the impulse activity in the control teeth was produced. Indomethacin produced a dose-dependent inhibition (ID_{50} 0.04–0.2 mg/kg). Diclofenac sodium (5 mg/kg) inhibited nerve activity by 51–91 % and naproxen (15 mg/kg) by 92–95 %. Methysergide (0.02–0.08 mg/kg) did not show such blocking effect. When the drugs were administered during a state of established increased neural activity no effect on the current impulse activity was obtained. The present results suggest that prostaglandins may be involved in certain stages of heat induced pulp inflammation and act as mediators of increased intradental sensory nerve excitability.

Pain produced by inflammatory processes is considered to be due to release of endogenous algogenic agents which then excite the nerve endings or increase their susceptibility to stimulation. Keele and Armstrong (1964, 1968) suggested that the increased receptor excitability is the most important factor in causing inflammatory pain. According to Skuteri (1968) increased sensitivity may be produced by 5-hydroxytryptamin and Olgart (1974) showed that this substance excites intradental nerves when applied to a dentinal cavity. Other substances which have been found in inflamed tissue and may be involved in the increase in the excitability are prostaglandins and their unstable intermediates (Vane 1972, Ferreira 1973). However, findings by Haegerstam and Edwall (1977) seemed to exclude a role of prostaglandins as mediators of pain in the dental pulp.

Recent investigations have shown that pathological alterations including increased sensory nerve activity can be induced in the dental pulp by means of repeated brief heat stimulation of the tooth surface (Ahlberg 1978). This method to induce and record an increased intradental neural activity provides a reproducible experimental model to study which algogenic

[illegible] agents are involved in producing such increased activity during inflammatory processes in [illegible] pulp.

The aim of the present study was to investigate whether non-steroid anti-inflammatory drugs, which interfere with prostaglandin synthesis and the serotonin blocking drug methysergide could block an experimental, heat-induced increase in sensory nerve activity in the dental pulp of the cat. Among the anti-inflammatory drugs indomethacin, diclofenac sodium and d-naproxen were selected for this study. These drugs have previously been shown to inhibit prostaglandin synthesis (Tomlinson *et al.* 1972, Vane 1972, Ku *et al.* 1975) and recent studies on renal prostaglandins indicate that diclofenac sodium might be used as a potent alternative to indomethacin (Oliw, Lundin and Änggård, in press 1978).

Material and Methods

The experiments were carried out on adult cats (2–5 kg) anaesthetized with chloralose (40 mg/kg i.) [illegible] urethane (50 mg/kg) or in some cases with sodium pentobarbital (30 mg/kg).

Immobilization and cavity preparation were performed as described earlier by Olgart, Haegerstam and [illegible] (1974). Electrical potentials from intradental sensory nerves were recorded from cavities extending [illegible] half the distance from enamel to pulp. The floor of the cavities [illegible] ere covered [illegible] a layer of amalgam [illegible] stainless steel tubes were secured by composite filling material as described by Edwall and Olgart [illegible]. Recording cables [illegible] ere connected to the tubes and conventional techniques were used to display the [illegible] and to store them on magnetic tape. The signals were counted on [illegible] scaler and printed out for 10 [illegible] and impulse frequency was given as impulses/s. A thermistor connected to a stainless steel tube [illegible] to the tooth surface afforded simultaneous registration of changes in the temperature.

[illegible] stimulation of the tooth surface by application for approximately 1 [illegible] of heated guttapercha at [illegible] C, produces an immediate increase in recorded neural activity which generally lasts 10–20 s. This first [illegible] may be regarded as [illegible] physical response to stimulation. When heat stimulations are repeated [illegible] subsequent more prolonged increase in nerve activity (phase 3) occurs after [illegible] latent interval (phase 2) following the phase 1 burst of impulses (Fig. 1 A).

This study [illegible] concerned with phase 3 as defined above. A quantitative expression of this activity was [illegible] by calculating the mean number of impulses per second over the period from 1–4 min inclusive [illegible] stimulation [illegible] excluding the first and second phases of the response. This parameter was reproducible [illegible] out for stimulation of different canine teeth prepared at the same time, variation being [illegible] within homologous pairs of teeth (Ahlberg 1978). The experimental procedure was carried out in the following way. After preparation each canine tooth [illegible] as stimulated with cold by applying [illegible] cotton pellet [illegible] in ethyl chloride to the tooth surface. This stimulus has been shown to elicit bursts of impulses [illegible] 1978) and was used as [illegible] test of the responsiveness to stimulation. The controls, generally one [illegible] and one lower canine tooth were selected by random choice. The control teeth were stimulated [illegible] with heat as described above until the maximum impulse activity was obtained. After intravenous injection of the drug to be tested, the procedure was repeated on the test teeth. Maximum phase 3 [illegible] were calculated for tests and controls and blocking effects of the drugs were expressed as per cent [illegible].

In another series of expts the blocking substance was given during [illegible] state of established heat induced [illegible] activity instead of as pretreatment.

The following drugs were tested.

Indomethacin, 1-(p-chlorobenzoyl)-5-methoxy-2-methylindole 3-acetic acid 0.04 [illegible] 0.2; 1 [illegible] 5 mg/kg b.w [illegible] 5 ml 0.1 mol sodium phosphate buffer pH 8.

Diclofenac sodium, 0-(2,6-dichlorophenyl-amino)-phenylacetate sodium 5 mg/kg b.w [illegible] in 5–7 ml isotonic saline solution.

Naproxen, d-2(6'-methoxy-2'-naphtyl-propionic acid) 15 mg/kg b.w in 9 ml 0.1 mol sodium phosphate buffer pH 8.

Methysergide [illegible] 9,10-didehydro-N(1-(hydroxy-methyl)propyl)-1,6-dimethylergoline-8-carboxamide 0.02–[illegible] mg/kg b.w [illegible] in 1–5 ml isotonic saline solution.

Unless specifically stated, drugs given as pretreatment were administered 30 to 60 min before stimulation. The rate of injection was about 1 ml in 10 s.

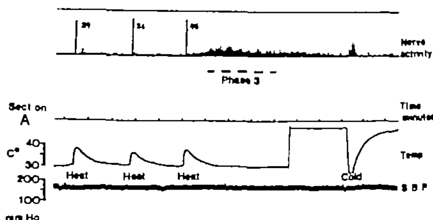


Fig. 1a

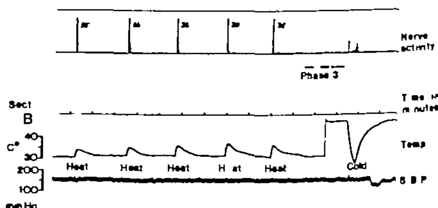


Fig. 1b

Fig. 1 Blocking effect by naproxen 15 mg/kg i.v on expected heat induced impulse activity Section shows recordings from the control tooth. In section B recordings from the test tooth are shown 25 mi after drug administration. Records are from above, intradental nerve activity time, temperature a systemic blood pressure. Temperature is registered t the tooth surface at 1/? crown length. Note the dr in base line between the last heat and the cold stimulation. Temperature calibration refers to the hea stimulations. The minimum temperature recorded during cold stimulation was about 10°C. Mean impuls frequency for period marked – – was in section A 17 6 imp/s and in section B 1.5 imp/s calculated from the scaler output.

Results

Blocking agents injected i.v as pretreatment effect on expected heat induced activity

Indomethacin, diclofenac sodium and naproxen were found to be potent inhibitors of expected increased sensory nerve activity Fig. 1 shows a typical protocol. An immediate response to heat stimulation occurred in the control tooth (Fig. 1 section A) and the first and second stimulations caused only a low phase 3 activity The third stimulation, however elicited marked impulse activity after a delay of about 1 min. The average number of impulses per second during this maximum phase 3 activity was 17 6. After about 6 min when the activity had largely subsided, cold stimulation caused a clearcut increase in impulse frequency In section B recordings from the test tooth are shown 25 min after naproxen 15 mg/kg i.v The immediate response to heat was the same as in the control tooth but the maximum phase 3 activity reached only 1.5 impulses per second despite repeated heat

Table I. Effect of pretreatment with drugs on phase 3 activity

Drug	Control		Pretreatment		Reduction per cent of control X̄ ± S.D.
	Number of teeth	Mean phase 3 activity imp/sec X̄ ± S.D.	Number of teeth	Mean phase 3 activity imp/sec X̄ ± S.D.	
Indomethacin [illegible] mg/kg	5	15.7 ± 4.6	7	2.0 ± 0.9	86 ± 7.9 p < 0.001
Diclofenac sodium 5 mg/kg	9	7.7 ± 2.9	13	2.0 ± 1.6	75 ± 12.3 p < 0.001
Naproxen [illegible] mg/kg	2	19.2 ± 2.3	2	1.3 ± 0.3	93 ± 2.1

Student's t test.

stimulation. Cold stimulation at the end of the section can be regarded as a test of the responsiveness of the tooth. No obvious changes in blood pressure were recorded following injection of the drug. The blocking effect as demonstrated here was a consistent finding in all experiments using indomethacin, diclofenac sodium and naproxen and the magnitudes of the reduction are shown in Table I.

The relationship of dose and the blocking effect of indomethacin was studied in 11 cats, 19 teeth. Doses from 0.04 to 5 mg/kg were administered, generally only one dose being given in each animal and the effects observed in one or two test teeth (3 and 7 cats respectively). In one cat two doses were given. The results are shown in Fig. 2. Blockade of neural activity was seen at the lowest dose studied (0.04 mg/kg) and ranged from zero to 36 per cent with a mean value of 15% (S.D. = ±17.5) of control. When the dose was increased to 0.2 mg/kg the blocking effect ranged from 72 to 88 per cent with a mean value of 81% (S.D ±7.0). A dose of 1 mg/kg resulted in a blocking effect of 91 per cent which was not increased by raising the dose to 5 mg/kg. In the cat given two doses the first dose (0.04 mg/kg) resulted

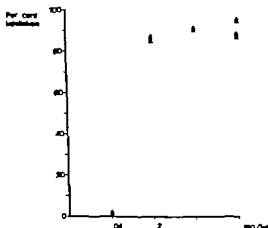


Fig. 2. Blocking effect by indomethacin 0.04–5 mg/kg i.v. on expected heat induced impulse activity. Inhibition expressed in per centage of control is marked on the ordinate.

in a blocking effect of 8.3 per cent. When the dose was increased to 0.2 mg/kg the block effect rose to 85 per cent as compared to control Data from this expt. are included in Fig

In similar expts, pretreatment with methysergide injected 3, 30, 60 or 90 min bef stimulation was ineffective in blocking the expected activity (5 teeth, 3 cats).

Blocking agents injected i.v. during established heat induced activity

In 15 cats the test drugs were given after the establishment of clearcut phase 3 activity alteration in this activity was seen with indomethacin (4 teeth), diclofenac sodium (4 tee naproxen (1 tooth) methysergide (8 teeth)

Discussion

The present study shows that indomethacin, diclofenac sodium and naproxen administer i v as a pretreatment could inhibit an expected, heat induced increased activity in the int dental sensory nerves. Methysergide did not show such blocking effect.

Indomethacin, diclofenac sodium and naproxen are characterized by their analges and anti-inflammatory properties (Flower 1974) It has been suggested that these effec are due to blockade of the prostaglandin synthesis (Vane 1972) and that the specific i hibitory action of the aspirin-like drugs is directed against the prostaglandin synthesis enzyme cyclooxygenase (Bergström 1977) Since this enzyme is found in most mammalia tissues it seems probable that it is present in the dental pulp also The blocking effect c indomethacin diclofenac sodium and naproxen on the heat induced sensory nerve activi seems to indicate that prostaglandins and/or their intermediates are involved in producin this increased neural activity It must be borne in mind, however that these compounds als exert inhibitory effects on other enzymes and cellular systems. The present finding that the blocking effect of indomethacin was dose dependent in the range 0.04–1 mg/kg supports the assumption that the effect was due to inhibition of prostaglandin biosynthesis since few of these other effects would be expected to occur at the low doses which were found in this study to inhibit sensory nerve activity (*cf* Flower 1974)

Methysergide blocks some 5-hydroxytryptamin receptors (Gaddum and Hameed 1954) but there is no evidence that it affects prostaglandin synthesis (*cf* Flower 1974) The findings that methysergide in pharmacological doses, which have been shown to block neural activity induced by compound 48/80 (Gazelius and Olgart 1977 unpublished data), had no inhibiting effect on expected, heat induced sensory nerve activity indicates that 5-hydroxytryptamin is not involved in producing such increase in intradental activity

The possible participation of prostaglandins in causation of increased intradental nerve activity has been studied by Haegerstam and Edwall (1977) using various ways to induce neural activity They found that activity induced by compound 48/80 applied to open cavities could not be blocked by indomethacin (5 mg/kg) or sodium acetylsalicylate (200 mg/kg) given i.v or (2.5 mg/ml and 9 mg/ml respectively) applied locally However the inhibitors of prostaglandin synthesis were given after the increased nerve impulse activity was established and this might in part explain their negative findings. They also found that in- ... at the tooth surface to 45–50°C in combination with close i.a.

infusion of prostaglandins or the PG-precursor arachidonic acid did not produce any neural activity. It seems likely that the heat stimulus was inadequate to produce inflammatory changes in the pulp.

Neither the immediate physical response to heat stimulation nor the response to cold were affected by pretreatment with indomethacin, diclofenac sodium or naproxen. These findings indicate that these drugs do not influence the excitability of the sensory nerves *per se* and furthermore, implies that prostaglandins are not essential to neural activity produced in normal, uninflamed tissue. This latter suggestion is supported by the finding reported by Haegerstam and Edwall (1977) that prostaglandin PGE_2 did not induce any nerve impulses when applied to a dentinal cavity.

The drugs, which were effective as pretreatment, were unable to influence a state of established increased activity. This may be a consequence of local ischemia at the site of the thermal injury preventing the drug from reaching the area where algogenic agents are present. This hypothesis is supported by recent findings that neural activity induced by a depot of compound 48/80 applied to a dentinal cavity could not be abolished by injection of tetrodotoxin or methysergide, whereas pretreatment with these drugs inhibited the activity (Gazelius and Olgart 1977, unpublished data). Since compound 48/80 probably causes reduced blood flow in the vicinity of the depot (Ahlberg and Edwall 1977) the lack of effect of the blocking agent might be due to incomplete distribution of the agent to the pulp under the cavity.

In conclusion, the results of the present study indicate that prostaglandins are involved in certain stages of heat induced pulp inflammation and that they in this situation act as mediators of increased activity in the sensory nerves of the pulp.

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Sympathetic control of metabolic and hormonal responses to exercise in rats

By

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Abstract

GALBO, H., E. A. RICHTER, N. J. CHRISTENSEN and J. J. HOLST *Sympathetic control of metabolic and hormonal responses to exercise in rats*. Acta physiol. scand. 1978. 102. 441–449

The importance of the sympatho-adrenal system for the pancreatic hormonal responses to exercise and, furthermore, the role of glucagon and catecholamines for the hepatic glycogen depletion during exercise was studied. Rats were either surgically adrenomedullectomized and chemically sympathectomized with 6-hydroxydopamine or sham-treated. Two weeks later the rats had either rabbit-antiglucagon serum or normal rabbit serum injected. Subsequently the rats either rested or swam with [illegible] tail weight for 75 min. Immediately afterwards cardiac blood was drawn and liver and muscle tissue collected. In control rats in spite of an increase in blood glucose concentrations during exercise plasma insulin concentrations were unchanged, while glucagon concentrations increased. In sympathectomized rats, compared to control rats, glucagon concentrations increased less, and insulin concentrations were higher although glucose concentrations were lower during exercise. Sympathectomy completely abolished the exercise-induced decrease in liver and muscle glycogen concentrations, whereas neither glycogen depletion nor plasma catecholamine concentrations were influenced by the administration of glucagon antibodies. These findings indicate that the sympatho-adrenal system enhances glucagon secretion as well as muscular and hepatic glycogen depletion but inhibits insulin secretion in exercising rats. The increase in glucagon concentrations, however, does not enhance hepatic glycogen depletion at the work load used.

Key words: Exercise, epinephrine, norepinephrine, glucagon, insulin, sympathetic nervous system, liver, muscle, glycogen, sympathectomy, adrenal medulla

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During prolonged exercise in man as well as in the rat the secretion of glucagon increases, whereas the secretion of insulin decreases. In both species the decrease in insulin secretion during exercise has been ascribed to a catecholamine mediated inhibition of pancreatic B-cell secretion (Galbo, Christensen and Holst 1977 c, Brisson, Malaisse-Lagae and Malaisse 1971). As to the regulation of glucagon secretion, however, in these two species different trends have been reported to be of major importance during exercise. In man the exercise induced increase in glucagon secretion could not be reduced by adrenergic blocking drugs (Galbo et al. 1977 a, 1977 c) but was markedly reduced by maintenance of euglycemia by

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Sympathetic control of metabolic and hormonal responses to exercise in rats

By

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Abstract

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The importance of the sympatho-adrenal system for the pancreatic hormonal response to exercise and, furthermore, the role of glucagon and catecholamines for the hepatic glycogen depletion during exercise was studied. Rats were either surgically adrenomedullectomized and chemically sympathectomized with 6-hydroxydopamine or sham-treated. Two weeks later the rats had either rabbit-antiglucagon serum or normal rabbit serum injected. Subsequently the rats either rested or swam with tail weight for 75 min. Immediately afterwards cardiac blood was drawn and liver and muscle tissue collected. In control rats in spite of an increase in blood glucose concentrations during exercise plasma insulin concentrations were unchanged, while glucagon concentrations increased. In sympathectomized rats, compared to control rats, glucagon concentrations increased less, and insulin concentrations were higher, although glucose concentrations were lower during exercise. Sympathectomy completely abolished the exercise-induced decrease in liver and muscle glycogen concentrations, whereas neither glycogen depletion nor plasma catecholamine concentrations were influenced by the administration of glucagon antibodies. These findings indicate that the sympatho-adrenal system enhances glucagon secretion as well as muscular and hepatic glycogen depletion but inhibits insulin secretion in exercising rats. The increase in glucagon concentrations, however does not enhance hepatic glycogen depletion at the work load used.

Key words: Exercise, epinephrine, norepinephrine, glucagon insulin, sympathetic nervous system, liver, muscle, glycogen, sympathectomy adrenal medulla

During prolonged exercise in man as well as in the rat the secretion of glucagon increases, whereas the secretion of insulin decreases. In both species the decrease in insulin secretion during exercise has been ascribed to a catecholamine mediated inhibition of pancreatic B-cell secretion (Galbo, Christensen and Holst 1977 c, Brisson, Malaisse-Lagae and Malaisse 1971). As to the regulation of glucagon secretion, however in these two species different results have been reported to be of major importance during exercise. In man the exercise-induced increase in glucagon secretion could not be reduced by adrenergic blocking drugs (Galbo *et al.* 1977 a, 1977 c) but was markedly reduced by maintenance of euglycemia by

glucose infusion during exercise (Galbo *et al.* 1977 a). These studies indicate that in man glucose availability is the major determinant of pancreatic A-cell secretion during exercise. From studies of immunosympathectomized rats (Luyckx *et al.* 1975) and of rats treated with adrenergic blocking drugs (Luyckx and Lefebvre 1974) it has been concluded that in the rat catecholamines play a major role in the exercise-induced glucagon secretion. Recently however, it has been shown that the glucagon as well as the catecholamine response to exercise are diminished in trained rats, which maintain high blood glucose concentrations for a longer period of time during exercise than untrained rats (Galbo *et al.* 1977 b). These findings are compatible with both the concept that glucose and the concept that catecholamines play the major role in exercise induced glucagon secretion.

It has been shown that the decrease in hepatic glycogen content elicited during prolonged heavy exercise in rats can be reduced by administration of glucagon-antibodies (Galbo and Holst 1976). This finding suggests that glucagon enhances hepatic glycogen depletion during exercise. However, also in antibody treated exercising rats a decrease in hepatic glycogen concentrations was observed. Accordingly it was concluded that other stimuli than glucagon, possibly catecholamines, may enhance hepatic glycogen depletion during exercise.

In the present study of rats, which were chemically sympathectomized and surgically adrenomedullectomized, we have tried further to evaluate the significance of the sympatho-adrenal system for the pancreatic hormonal secretion during exercise. Furthermore, by administration of glucagon-antibodies to sympathectomized as well as to control rats we tried to evaluate the relative role of glucagon and catecholamines for the hepatic glycogen depletion during exercise.

Material and methods

69 male Wistar rats weighing 230–250 g were anesthetized with ether and either adrenodemedullated or sham operated. Adrenodemedullation was performed by electrocoagulation through bilateral lumbar incisions. The first week after the operation the rats had free access to 0.9 % [g (100 ml)$^{-1}$] NaCl as drinking water. In order to allow recovery of cortical tissue the surgery was carried out 15 days prior to the exercise test.

In adrenodemedullated rats chemical sympathectomy was accomplished according to the procedure, which has been found to result in the most complete destruction of adrenergic nerve terminals in rats (Thoenen and Tranzer 1968). Two days after surgery the animals received (in a tail vein and six hours apart) two injections of 34 mg kg b.wt.$^{-1}$ 6-hydroxydopamine dissolved immediately before injection in 0.5 ml 0.9 % NaCl containing 0.5 mg ascorbic acid. One week later the rats received further 2 × 68 mg kg^{-1} 6-hydroxydopamine I. Sham operated rats had only the solvent injected.

The exercise test was carried out in the morning, and the rats fasted 12 h in advance. During light ether anesthesia 0.3 ml of blood were drawn by cardiac puncture for glucose analysis and 0.5 ml of either rabbit-antiglucagon serum or normal rabbit serum were injected through the cardiac cannula. After 15 min of recovery the rats were either forced to swim with a tail weight of lead (2 % of b.wt.) in water maintained at 33–34°C for 75 min, or they remained resting. The number of rats assigned to the various procedures appear from the figures and tables. At the end of exercise or equivalent period of rest, the rats were superficially anesthetized with ether and 8 ml of blood were drawn by cardiac puncture. Then samples of the liver, of the superficial part of the vastus lateralis muscle (which consists predominantly of fast-twitch white fibers), of the deep part of the vastus lateralis muscle (predominantly fast twitch red fibers), of the soleus muscle (predominantly slow-twitch red fibers) and of the pancreas were quickly removed and frozen in liquid nitrogen. In sympathectomized rats the integrity of the adrenal cortex and the absence of chromaffine cells were verified by light microscopy of two sections, stained by hematoxylin and eosin, of each adrenal gland.

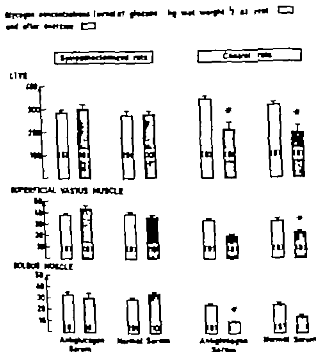


Fig. 1. The effect of 75 min of forced swimming on the concentrations of glycogen in the liver and in two muscles with different fiber types. Sympathectomized rats were adrenodemedullated and treated with 6-hydroxydopamine two weeks before sacrifice. Antiglucagon serum or normal rabbit serum was injected immediately prior to exercise or equivalent period of rest. Values are mean ± S.E. Number of observations is shown in parenthesis. Asterisk signifies $P < 0.05$ for difference between values at rest and after exercise. Resting muscular glycogen concentrations were significantly higher ($p < 0.05$) in sympathectomized than in control rats.

The methods of analysis of blood and tissue for hormones and metabolites have been reported elsewhere (Galbo and Holst 1976, Galbo *et al.* 1977 b). All analyses were performed at least in duplicate and all radioimmunological determinations of [illegible] hormone were carried out in [illegible] single assay run. Tissue samples were stored at −80°C and analyzed in random order. Glycogen concentrations were expressed as mmol of glucose · kg wet tissue⁻¹. The precision of the glycogen determinations calculated as the coefficient of variation was 7 [illegible] at 34 mmol kg⁻¹ for double determinations of nine samples (4–14 mg) of the same muscle, and 4 [illegible] at 300 mmol kg⁻¹ for double determinations on 6 samples from the same liver. The characteristics of the antiglucagon serum that was administered to the rats have been described in detail elsewhere (Holst, Galbo and Richter 1978). At sacrifice 300 µl of 1 : 1 400 diluted plasma from rats treated with antiglucagon serum were able to bind 40 % of 10 fmol 125-I-labelled glucagon. Glucose concentrations were compared by the t-test for paired comparisons, and catecholamine concentrations by the t-test for unpaired comparisons (Snedecor and Cochran 1965). The remaining data were subjected to analysis of variance preceded by Bartlett's test, and significant differences were located by Tukey's test (Snedecor and Cochran 1965).

Results

In control rats treated with normal serum glycogen concentrations in liver as well as in skeletal muscle had significantly decreased after 75 min of swimming (Fig. 1 and Table III). Simultaneously the concentrations in plasma of glucagon, glucose, lactate and pyruvate

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Table I. Tissue catecholamine concentrations (ng·g⁻¹ wet weight) in sympathectomized (SX) and control rats (C).

	Pancreas		Liver		Deep vastus muscle	
	SX	C	SX	C	SX	C
Norepinephrine	32.3±7.5* (9)	239.0±27.0 (7)	1.1±0.4 (8)	24.2±2.5 (8)	21.8±2.5 (9)	81.2±6.0 (8)
Epinephrine	0.6±0.5 (8)	14.7±2.1 (7)	0.6±0.6 (7)	0.2±0.2 (7)	4.5±1.1† (8)	1.0±0.4 (8)

Values are mean ± S.E. Number of observations are shown in parenthesis. Samples of the pancreas were obtained from exercised rats, whereas liver and muscle samples were obtained from resting rats. * ($p<0.001$) and † ($p<0.01$) denote that SX rats differ significantly from C rats.

$p<0.05$, n = 7) but not in sympathectomized rats. In resting rats sympathectomy significantly increased the concentrations of muscle glycogen (Fig. 1 and Table III) and significantly decreased the concentration of glucagon in plasma (Fig. 2). During exercise neither the concentrations of glycogen in liver[1] and muscle (Fig. 1 and Table III) nor the concentration of glucose in blood (Fig. 2) changed in sympathectomized rats. Furthermore, during exercise the concentration of glucagon in plasma increased less in these rats than in control rats (Fig. 2). The concentration of insulin in plasma was higher in exercised sympathectomized rats than in both exercised and resting control rats (Fig. 2). The responses of the remaining variables to exercise were not influenced by sympathectomy.

Effects of antiglucagon serum

In resting rats the concentration of alanine in plasma was increased by the administration of antiglucagon serum in control as well as in sympathectomized rats (Table III). Apart from that, comparisons of rats treated with antiglucagon serum with rats treated with normal serum did not reveal any consistent differences. Thus, the plasma concentrations of catecholamines were not altered by antiglucagon serum (Table II).

Table II. Plasma concentrations (ng·ml⁻¹) of norepinephrine and epinephrine in rats after 75 min of swimming.

	Sympathectomized and glucagon-depleted	Glucagon-depleted	Controls
Norepinephrine	0.92±0.09* (7)	3.48±0.84 (7)	3.14±0.59 (7)
Epinephrine	0.36±0.12* (7)	1.52±0.39 (7)	1.40±0.32 (7)

Values are mean ± S.E. Number of observations are shown in parenthesis. Glucagon depleted rats had been treated with antiglucagon serum and controls with normal rabbit serum. * denotes that the sympathectomized group of rats differed significantly ($p<0.02$) from the other groups.

[1] The lack of decrease of hepatic glycogen concentrations was verified by reanalysis of all liver samples.

The conc ntrations of glucose (mmol l) blood and of ulin (pmol l⁻¹) and glucagon (pmol l⁻¹) erum at rest ☐ and fter ex cise ☐

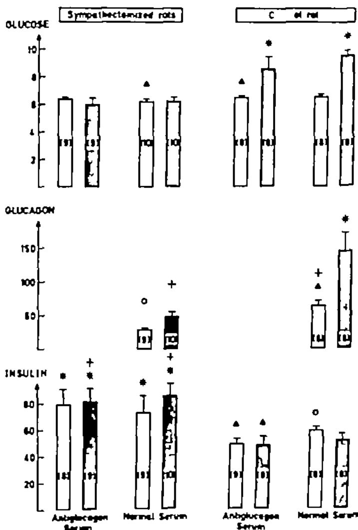


Fig. 2. Values are mean ± S.E. Number of observations are shown in parenthesis. values are significantly higher than ^values of the same variable ($p < 0.05$), and +values are significantly higher than °values ($p < 0.05$). Blood for glucose analysis was obtained from each rat before as well as after exercise, whereas blood for hormone analyses was obtained only after exercise or equivalent period of rest. For further explanation see Fig. 1

had increased (Fig. 2 and Table III), whereas the plasma concentrations of FFA, glycerol, alanine and insulin did not change significantly During exercise in control rats glucagon concentrations correlated significantly with norepinephrine ($r = 0.91$ $p < 0.01$ $n = 7$) as well as with epinephrine ($r = 0.77$ $p < 0.05$ $n = 7$) concentrations in plasma. In none of the resting groups of rats did the blood glucose concentration change significantly during the 90 min rest period (not shown).

Effects of sympathectomy

The concentrations of catecholamines in pancreas, liver and skeletal muscle (Table I) as well as in plasma (Table II) were significantly diminished by the applied combination of chemical sympathectomy and adrenodemedullation. The concentrations of epinephrine and norepinephrine in plasma correlated significantly during exercise in control rats ($r = 0.87$

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Effects of antiglucagon serum

In resting rats the concentration of alanine in plasma was increased by the administration of antiglucagon serum in control as well as in sympathectomized rats (Table III). Apart from this, comparisons of rats treated with antiglucagon serum with rats treated with normal serum did not reveal any consistent differences. Thus, the plasma concentrations of catecholamines were not altered by antiglucagon serum (Table II).

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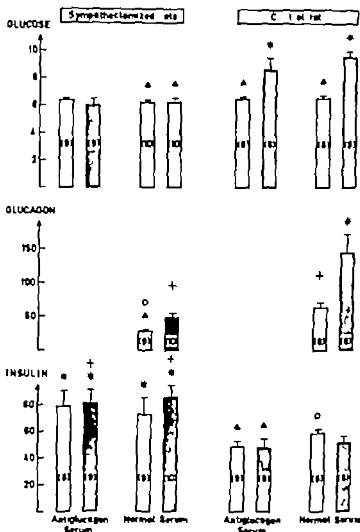


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of less efficient sympathectomy procedures (Gollnick *et al.* 1970, Maling *et al.* 1966, Sembrowich *et al.* 1974) or of less precise techniques for the determination of glycogen (Maling *et al.* 1966), in the lack of appropriate control experiments (Gollnick *et al.* 1970, Sembrowich 1974), in the use of trained animals (Gollnick *et al.* 1970, Sembrowich *et al.* 1974) or in the employed exercise procedures.

It has been repeatedly observed that glycogen depletion can be elicited in isolated, perfused skeletal muscle by electrical stimulation (Berger *et al.* 1975, Chapler and Stainsby 1968, Costin *et al.* 1970, Helmreich and Cori 1966, Karlsson, Rosell and Saltin 1972). Furthermore, the capacity for work performance is reduced in patients with McArdle's disease, in whom muscular glycogenolysis is inhibited by a deficiency of glycogen phosphorylase (Pearson, Rimer and Mommaerts 1961). At first thought our present finding that sympathectomized animals were able to exercise for a prolonged period without a fall in muscle glycogen may seem incompatible with these facts. But catecholamines may be present during perfusion of skeletal muscle preparations either as a consequence of excitation of sympathetic nerve fibers within the electrically stimulated nerve or due to the use of blood as the perfusion medium. Furthermore, insufficient supply of oxygen or free fatty acids to the perfused contracting muscle due to insufficient perfusate flow or to the use of artificial perfusates may enhance glycogenolysis and thus reduce the applicability of findings in isolated preparations to the interpretation of findings in intact exercising animals. As to the limited work tolerance in patients suffering from McArdle's disease it should be remarked that these patients tolerate low work intensities indefinitely (Pearson *et al.* 1961). It is probably so that very low work loads normally can not elicit muscular glycogenolysis (Chapler and Stainsby 1968). A range of higher work loads including the one used in the present study may be characterized by the existence of a catecholamine facilitated muscular glycogenolysis during physiological conditions and by the ability of the working muscles to derive the necessary energy from alternative sources thus performing equally well when glycogenolysis is impeded. In accordance with these considerations pretreatment of an isolated muscle with epinephrine in concentrations, which per se does not produce a rise in the concentration of active phosphorylase, causes an augmented increase in the concentration of this enzyme during subsequent electrical stimulation (Helmreich and Cori 1966). Furthermore, beta adrenergic blockade has been shown to diminish the reduction in glycogen content in an in situ isolated, stimulated muscle preparation (Costin *et al.* 1971). The possibility that breakdown of other substrates may substitute for glycogen breakdown during prolonged exercise is supported by the findings of the present study as well as of the ability of a fat emulsion to improve exercise tolerance in patients suffering from McArdle's disease (Pearson *et al.* 1961) and of the ability of working muscles to utilize endogenous triglycerides during prolonged exercise (Reitman, Baldwin and Holloszy 1973).

As to the pancreatic hormonal response to exercise we found that in control rats, in spite of an increase in blood glucose concentrations, plasma insulin concentrations were unchanged while glucagon concentrations increased during exercise. In sympathectomized rats no such exercise-induced inhibition of insulin secretion was found, and the exercise-induced enhancement of glucagon secretion was much less prominent (Fig. 2). I these rats compared to control rats glucagon concentrations increased less and insulin concentrations were

TABLE III The concentrations in serum (FFA and glycerol), blood (lactate and pyruvate), plasma (ala and in the deep part of the vastus lateralis muscle (glycogen) of substrates in rats at rest after 75 min of swimming. The rats were either sympathectomized (SX) or controls (C) either treated with antiglucagon serum or normal rabbit serum.

	FFA meq l⁻	Glycerol mmol l⁻	Lactate mmol l⁻	Pyruvate μmol l⁻	Alanine μmol l⁻¹	Glycog mmol l⁻
C-rats, normal serum Rest	1.325±0 186 (8)	0 473±0.079 (8)	2.66±0.67 (8)	68±12 (8)	392±15 (7)	33±2 (8)
C-rats, normal serum After exercise	1 151±0.226 (8)	0 427±0.040 (8)	5.69±0 63 (8)	136±24 (8)	353±21 (8)	19±2 (8)
C rats, antiglucagon serum Rest	1.278±0.284 (9)	0 406±0.105 (9)	1 71±0 09 (9)	74±10 (9)	471±36 (8)	32±2 (9)
C rats, antiglucagon serum After exercise	1.397±0.306 (6)	0.568±0.104 (6)	5.32±1.20 (8)	163±23 (8)	366±21 (8)	19±2 (8)
SX-rats, normal serum Rest	0 644±0.080 (9)	0 297±0.054 (9)	1 69±0.17 (9)	56±14 (8)	420±18 (8)	40±3 (9)
SX rats, normal serum After exercise	0 799±0 063 (9)	0.373±0.036 (9)	6.99±1.97 (8)	126±.8 (8)	352±18 (9)	40±2 (10)
SX-rats, antiglucago serum Rest	1.366±0.190 (8)	0.534±0.109 (8)	1.54±0 08 (8)	53±12 (8)	480±32 (8)	40±2 (8)
SX rats, antiglucagon serum After exercise	1.042±0.123 (8)	0.562±0.063 (7)	4 62±1 13 (8)	103±15 (8)	430±22 (8)	38±4 (8)
Least significa t difference	0 455	0 184	2.26	43	57	6

Values are mean ± S.E. N mber of observations are shown i parentheses. The least significant differe ($p < 0.05$) between two mean values of the same variable was calculated by analysis of variance and Tuk test (Snedecor and Cochran 1965).

Discussion

The present study has demonstrated a remarkable influence of sympathectomy on metabol and hormonal responses to exercise. In contrast to findings in control rats neither hepat nor muscular glycogen concentrations decreased in sympathectomized rats during exercis Previous studies on the contribution of the sympatho-adrenal system to the glycogen deple tion in liver and skeletal muscle during exercise have given inconsistent results. Certainly although less pronounced compared to the one in the present study an inhibition of exercise induced glycogen depletion has been found in some studies of adrenomedullectomized rat (Sembrowich *et al.* 1973 Struck and Tipton 1974) and of rats adrenomedullectomized as well as chemically sympathectomized (Maling *et al.* 1966). But other studies of adrenomedul lectomized rats (Gollnick *et al.* 1970, Maling *et al* 1966) and of rats adrenomedullectomized as well as chemically sympathectomized (Gollnick *et al.* 1970 Sembrowich *et al* 1973) failed to demonstrate any inhibition of glycogenolysis. The reasons why these studies in contrast to the present study did not convincingly reveal the significance of an intact sympatho- adrenal system for the glycogen depletion during exercise are likely to be found in the use

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higher in the face of lower blood glucose concentrations during exercise. These finding which could not be ascribed to differences in plasma levels of FFA and alanine (Table III indicate that in the rat sympathetic discharge significantly promotes glucagon secretion and inhibits insulin secretion during prolonged exercise. Similar conclusions have previously been drawn from studies of rats which before exercise had been either immunosympathectomized, adrenomedullectomized or treated with adrenergic blocking agents (Brisson, Malaisse Lagae and Malaisse 1971 Luyckx and Lefebvre 1974 Luyckx *et al* 1975). Since in man catecholamines probably account to only a minor extent for the glucagon response to prolonged exercise (Galbo *et al* 1977 a, 1977 c), a species difference as regards the regulation of glucagon secretion during exercise seems to exist between man and rat.

The lower concentration of glucagon in plasma during exercise after sympathectomy hardly explained the lack of hepatic glycogen depletion in sympathectomized rats. This is so since in glucagon depleted control rats the rate of hepatic glycogen depletion in the absence of a compensating catecholamine stimulus (Table II) were the same as in control rats treated with normal rabbit serum. This apparent lack of influence of glucagon on hepatic glycogen mobilization in exercising rats is inconsistent with the conclusion in a previous study in which hepatic glycogen depletion in control rats was larger than in the present study (Galbo and Holst 1976). However during exercise increased glucagon secretion possibly only enhances hepatic glycogen mobilization at high rates of glycogen breakdown or at reduced glycogen levels. Alternatively the lack of decrease of plasma insulin concentrations during exercise found only in the present study accounts for the discrepancy since insulin deficiency makes the liver more sensitive to the glycogenolytic effect of glucagon (Mackrell and Sokal 1969 Wagle 1975).

The present study has shown that the pancreatic hormonal response as well as muscular and hepatic glycogen depletion during prolonged exercise are markedly reduced in sympathectomized rats compared to control rats. These findings indicate that the sympatho-adrenal system enhances glucagon secretion and glycogen depletion but inhibits insulin secretion in normal exercising rats. The contributions of sympathetic nervous activity and of adrenomedullary secretion to these effects still have to be settled Neither has the present study established whether catecholamines directly enhance glycogen breakdown and glucagon secretion during exercise or whether these effects are due to a primary inhibition of insulin secretion (Brisson *et al* 1971 Galbo *et al* 1977 c, Luyckx and Lefebvre 1974 Porte 1967) insulin being able to inhibit glucagon secretion (Charles *et al* 1976) and to promote glycogen storage (Berger *et al.* 1975 Craig, Rall and Larner 1969 Mackrell and Sokal 1969 Miller and Larner 1973)

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to fluid mobilization from the extra- to the intravascular space (Lundvall and Hillman, published). The described findings suggest that neurogenic and humoral microvascular energic dilator effects, if evoked during integrated reflex activation of the sympatho- al system, might be of considerable importance for transcapillary hydrodynamic nge.

hypothesis was tested in the present study in which the sympatho-adrenal system was ated by hemorrhage. During bleeding, transcapillary absorption of fluid into the blood constitutes an important mechanism for refill of the circulatory system and thereby for ration of cardiovascular homeostasis. Such fluid transfer from the extravascular to the vascular space mainly occurs in skeletal muscle, which, due to its large tissue mass, its considerable volumes of fluid to be mobilized into the circulation. The fluid absorp- from skeletal muscle during bleeding seems mediated mainly by two mechanisms, i.e. by adrenergic resetting of the pre- to postcapillary resistance ratio, leading to fluid ab- tion via a decrease of capillary hydrostatic pressure (e.g. Öberg 1964), and by osmotic absorption caused by arterial hyperosmolality due to glucose release from the liver et al. 1972, Järhult 1973). The relative importance of these two mechanisms has not been more precisely defined. There is experimental evidence to indicate, however that direct adrenergic mechanism is the more important one in early stages of bleeding, cially at small and moderate blood loss (Öberg 1964), whereas during more prolonged severe hemorrhage the glucose-osmotic fluid absorption seems to be at least equally ortant (Järhult 1973, Järhult et al. 1976). In the present investigation, in which emphasis placed on the direct adrenergically evoked fluid mobilization from skeletal muscle during ding, the animals were exposed to a relatively small hemorrhage (withdrawal of 15% of blood volume) and the period of hemorrhagic hypotension was limited (10 min) to imize activation of the glucose-osmotic fluid absorption mechanism.

Methods

ats of both sexes, weighing 2.6–3.9 kg were used in the study. The animals were anesthetized with alose (50 mg/kg b.wt.) and urethane (100 mg/kg b.wt.) after induction with ether. Body temperature maintained at 38±0.5°C throughout the experiment.

he investigation was performed on the innervated vascular bed of the lower leg muscles, isolated so that popliteal artery and vein formed the sole vascular connections with the main part of the animal. In most animals the muscle preparation was made on the right lower leg only. In some of the animals, also the lower leg muscles were isolated so that simultaneous observations could be made on two separate cle regions. The muscle preparation was placed in a water-filled plethysmograph (38°C) to permit tinuous recording of tissue volume (for surgical and technical details, see Lundvall 1972). After heparin- on (3–5 mg/kg b.wt.), the popliteal vein was cannulated and the venous outflow from the region shunted tubes to a funnel connected to the right jugular vein. An optical drop recorder unit was inserted in the ous shunt for continuous recording of blood flow. The arterial inflow pressure was measured from the brachial artery. The right brachial artery was cannulated to permit bleeding and withdrawal of arterial d for determination of plasma osmolality and plasma glucose concentration. Sampling of venous blood osmolality and glucose determination was made via a T-tube in the venous shunt close to the cannulated liteal vein. Plasma osmolality was measured by thermistor cryoscopy (Osmometer 31 LAS, Advanced truments, Inc.) and plasma glucose concentration with the glucose-oxidase method. Blood flow and rial blood pressure, the latter measured with a Statham transducer, were recorded on a Grass Polygraph. The reactions of the resistance vessels in the muscle caused by bleeding were obtained from the regional rio-venous pressure difference and blood flow. Net transcapillary fluid movements were determined

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Fluid transfer from skeletal muscle to blood during hemorrhage

Importance of beta adrenergic vascular mechanisms

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Abstract

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Vascular reactions in the cat lower leg muscles in response to short term (10 min) hemorrhagic hypotension (~80 mmHg) were studied before and after regional blockade of the β-adrenoceptors. In the muscle region with intact β-adrenoceptors, hemorrhage raised vascular resistance by about 80% and caused a dilatation of the precapillary sphincters, the latter effect evidenced in terms of a 35% increase of the capillary filtration coefficient. Concomitantly an absorption of extravascular fluid to the blood stream occurred, a process tending to compensate for the reduction of intravascular fluid volume. After regional β-blockade there is quite a marked augmentation of the hemorrhage induced increase of vascular resistance whereas the inhibition of precapillary sphincter tone and the transcapillary fluid absorption were almost abolished. The observations indicate that bleeding is associated with a significant β-adrenergic dilator influence in both the resistance vessels and precapillary sphincters of skeletal muscle and that the β-dilator mechanism may be essential for the important, compensatory fluid gain from the extravascular to the intravascular space during hemorrhage. The observed β-adrenergic mediation of the net transcapillary fluid absorption could be ascribed to resetting of the pre-/postcapillary resistance ratio, leading to decreased capillary hydrostatic pressure, and to the dilator influence in the precapillary sphincters, leading to an increased number of the patent capillaries available for the transcapillary fluid exchange.

Recent studies from this laboratory have demonstrated that electrical activation of the sympathetic vasomotor fibres to skeletal muscle is associated with a β-adrenergic dilator response in resistance vessels of small diameter and in precapillary sphincters (Lundvall and Järhult 1974 1976) The β-adrenergic dilator component of the sympathetic vascular response was found to markedly counteract the α-adrenergic constriction in the microcirculation and to significantly augment the net transcapillary absorption of extravascular fluid induced by sympathetic stimulation. The facilitation of the fluid transfer could be ascribed partly to the inhibition of precapillary sphincter tone, leading to an increased number of patent capillaries available for transcapillary exchange, and partly to a reinforcement of the nerve induced fall of capillary hydrostatic pressure. Intra-arterial infusion of adrenaline and noradrenaline was found to cause similar preferential microvascular β-adrenergic dilator effects contrib-

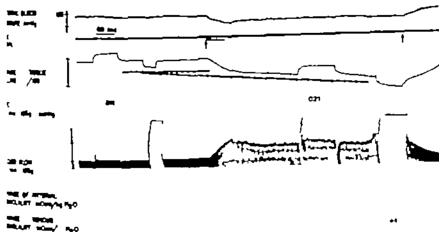


Fig. 1. Original record illustrating the pattern of vascular responses evoked by bleeding. Onset of hemorrhage and of reinfusion of blood indicated by arrows. Changes of plasma osmolality given at bottom of the record. For detailed description see text.

approximately steady state level of 3.7 ml/min · 100 g. The flow reduction was partly due to the pressure fall and partly to an increase of regional vascular resistance, which was raised from the control value of 11.6 to 21.4 mmHg/(ml/min · 100 g) in the steady state period of bleeding. Tissue volume, being approximately constant in the control period (isovolumic state), showed quite a pronounced decrease initially during hemorrhage reflecting mobilization of regional blood (capacitance response). The subsequent slow and continuous decline of tissue volume, more clearly visualized by the broken lines drawn beneath the volume recording, represents net transcapillary fluid absorption, in this experiment occurring at a rate of 0.055 ml/min · 100 g tissue. The CFC values obtained in the record indicate that bleeding was associated with a relaxation of the precapillary sphincters, which increased the functional capillary surface area for fluid absorption by some 50% (*cf.* Methods). The figures for plasma osmolality at bottom of the record, finally, show that bleeding caused some increase (3 mOsm/kg H_2O) of arterial osmolality and that venous osmolality also tended to increase.

Regional blockade of the β-adrenoceptor was found to cause quite marked quantitative alterations of the described vascular responses evoked by hemorrhage. The resistance increase evoked during bleeding was thus always greater after β-blockade, whereas the inhibition of precapillary sphincter tone and the net transcapillary fluid absorption instead was much reduced. These effects are summarized in Fig. 2 (mean values ± S.E.). Panel A shows that vascular resistance in the intact region was raised during hemorrhage by 77% above the resting control level whereas in the β-blocked region the corresponding resistance increase averaged 112% ($p < 0.05$). This augmentation after β-blockade indicates the presence during hemorrhage of a β-adrenergic dilator influence, strong enough to significantly counteract the simultaneous constrictor response in the resistance vessels. The changes of arterial blood pressure during bleeding were quite similar before and after β-blockade;

from observed alterations of tissue volume and could be separated from capacitance effects in the vasc. bed, since the latter develop rapidly and in coordination with the effects in the resistance vessels (see Mellander 1960, Kjellmer 1965). Precapillary sphincter activity determining the number of patent capillaries and, hence, the functional capillary surface area available for transcapillary exchange, was followed by measurement of the capillary filtration coefficient (CFC). CFC was determined from the tissue volume recording by calculating the rate of net transcapillary fluid filtration caused by raising venous outflow pressure (VP) 5 mmHg, assuming that 80% of the venous pressure rise was transmitted to the capillary level (Cobbold *et al* 1963). Venous pressure was changed by adjustment of the height of the orifice of the outflow tubing from the muscle region. Except during the CFC determination, venous pressure was maintained at a normal level (4–6 mmHg), which gave a constant tissue volume in the control period (isovolumic state).

When the surgical procedures were finished the animals rested for 30 min before experimental intervention. Hemorrhage was performed by bleeding the animal into a graded syringe so that 15% of the calculated blood volume was withdrawn within 1 min (blood volume considered to be 7% of the body weight, Altman and Dittmar 1971). After 10 min of hemorrhage the shed blood was reinfused. Regional β-adrenoceptor blockade was produced by administration of propranolol (600 μg/kg muscle) close arterially via a cannula inserted into a side branch of the femoral artery. The venous outflow was discarded during the administration of propranolol to avoid systemic effects. Test experiments in which the dextro-isomere of propranolol was used showed that the reported effects of the blocking agent were not related to a nonspecific influence, such as an anesthetic one.

In Results section, spread of data is expressed as S.E. Significance tests were performed according to Student's t test.

Results

The effect of regional blockade of the β-adrenoceptors on the vascular responses evoked by hemorrhage in the innervated lower leg muscle preparation was studied in 9 animals. In 6 of the animals observations were made on the vascular bed of the right lower leg muscles only. The vascular changes induced by standardized bleeding (withdrawal of 15% of the blood volume for a period of 10 min) were here first studied when the β-adrenoceptors were left intact (below denoted "intact" region). After reinfusion of the shed blood, regional blockade of the β-adrenoceptors was performed and the hemorrhage experiment repeated after a 2[illegible] min period of rest. Separate control experiments on 4 animals in which no β-blockade was performed showed that repetetive bleeding as such evoked reproducable changes. In the other 3 animals in which the effect of β-blockade was studied, the lower leg muscles were isolated on both sides and after the 30 min period of rest after completion of surgery propranolol was given close arterially to the right leg muscles whereas the left leg muscle preparation was left intact. This experimental approach permitted simultaneous observations of the vascular responses in the intact and in the β-blocked muscle region during hemorrhage. Two periods (10 min) of bleeding with an interval of 20–30 min were performed on each of these 3 animals. The results from the described two groups of animals in which β-blockade was performed were quite similar and will be presented together.

Fig. 1 showing tracings of mean arterial blood pressure, muscle blood flow and tissue volume, illustrates the typical pattern of vascular response evoked by hemorrhage in the intact muscle region. The capillary filtration coefficient (CFC) and arterial and venous osmolality were determined before and during bleeding. It can be seen that hemorrhage caused a rapid decrease of arterial blood pressure down to an initial, transient low level (65 mmHg), after which pressure increased somewhat and stabilized on a hypotension level (~80 mmHg) maintained throughout the period of bleeding. Concomitant to the pressure decline, blood flow decreased rapidly from the control value of 8.8 ml/min 100 g to an

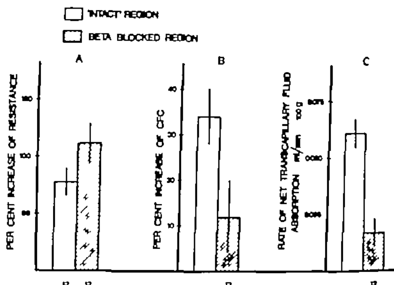


Fig. 2. Diagrams showing the effects of bleeding on vascular resistance (Panel A), on CFC, reflecting pr capillary sphincter tone (Panel B), and on net transcapillary fluid absorption (Panel C) before and aft β-blockade. The data shows that β-blockade augmented the resistance increase during hemorrhage where the precapillary sphincter dilatation and transcapillary fluid transfer were markedly reduced. The conclusio was reached that hemorrhage is associated with a β-adrenergic dilator influence in the resistance vessels a precapillary sphincters and that the β-dilator effects are essential for the gain of extravascular fluid to th blood stream.

control blood pressure averaged 117 mmHg in the intact region and 118 mmHg in the β blocked region and the steady state hemorrhage hypotension averaged 77 and 75 mmHg respectively. Resistance changes due to passive elastic recoil of the vascular wall as transmura pressure decreased must therefore have been in the same order of magnitude in the tw situations.

CFC in the resting control period averaged 0.013 ± 0.001 (n = 12) ml/min 100 g mmHg in the intact region and 0.015 ± 0.001 (n = 12) ml/min 100 g mmHg in the β-blocked region. Panel B in Fig. 2 shows the mean per cent increase of CFC above control during bleeding. It can be seen that in the intact region, CFC was raised by 34%. This effect, reflecting a relaxation of the precapillary sphincters, seems to a great extent caused by β-adrenoceptor activation since the increment of CFC in response to hypovolemia in the propranolol treated muscle was much smaller (12%, $p < 0.01$).

Diagram C in Fig. 2 depicts the rate of net transcapillary fluid absorption during hypovolemia. The data refer to observations 8–9 min after commencement of bleeding but are representative for the whole period of bleeding since the absorption rate was roughly constant in the intact (*cf.* Fig. 1) as well as in the β-blocked region. In the intact muscle region, the rate of fluid transfer averaged 0.062 ± 0.006 (n = 12) ml/min 100 g tissue implying that a total of some 0.60 ml/100 g tissue of extravascular fluid was mobilized into the blood stream during the 10 min period of hypovolemia. The observed fluid transfer can mainly be ascribed to a reflex decrease of capillary hydrostatic pressure since the increment of arterial blood osmolality during bleeding, leading to osmotic fluid gain into the circulatory system, was quite small (see Discussion). Arterial plasma osmolality was raised by 4 mOsm/kg H_2O on an

average towards the end of the period of hemorrhage. Concomitantly venous osmolality had increased by a mean value of 2 mOsm/kg H_2O. The determinations of plasma glucose concentration indicated that both the arterial and venous osmolar increase almost entirely was due to hyperglycemia (1 mM/L of glucose considered to correspond to 1 mOsm/kg H_2O), a finding in agreement with previous reports (Järhult 1973, Järhult *et al* 1976).

In the β-blocked muscle region, the rate of fluid absorption was much reduced compared to that in the intact region ($p < 0.001$) and it here averaged only 0.017 ± 0.005 ($n = 12$) ml/min · 100 g tissue (corresponding to a total amount of mobilized fluid during the 10 min of bleeding of about 0.15–0.20 ml/100 g tissue). This reduction of the fluid transfer after β-blockade can hardly be related to the glucose-osmotic mechanism since the observed alteration of blood osmolality during bleeding was similar to that in the experiments in which the β-adrenoceptors in the muscle region were left intact. Arterial osmolality thus showed an average increase of 3 mOsm/kg H_2O during bleeding and venous osmolality concomitantly rose by 1 mOsm/kg H_2O; as in the intact region, the osmolar change could be ascribed to an increase of the plasma glucose concentration. The effects of propranolol on the fluid transfer during hemorrhage instead seems related to blockade of regional, vascular β-adrenergic mechanisms in the muscle which, in the intact region, facilitate transcapillary fluid mobilization. Beta-adrenergic relaxation of precapillary sphincter tone, increasing the number of patent capillaries and thereby the capillary surface area available for transcapillary fluid exchange, seems to be one such effect as indicated by the above described CFC data.

Discussion

The present study strongly suggests that bleeding is associated with a β-adrenergic dilator influence in the skeletal muscle vascular bed. This was revealed in terms of a significantly higher vascular tone in both the resistance vessels and in the precapillary sphincters in the β-blocked muscle region than in the muscle region with intact β-adrenoceptors during standardized hemorrhagic hypotension. It should be stressed that the alterations of the vascular responses to bleeding evoked by β-blockade entirely must reflect a regional effect in the studied muscle of the β-blocking agent. The β-blocking agent was thus administered locally by close arterial infusion and concomitantly the venous effluent was sampled and discarded. Interaction of the remote nervous and hormonal control of the muscle vascular bed due to, for example, blockade of β-adrenoceptors in the central circulation or possible interference with autonomic circulatory control centres was thereby avoided.

As a result of the β-adrenergic dilator influence, the net resistance increase in the muscle during bleeding was reduced by about 30%. In absolute resistance units, the β-adrenergic effect amounted to some 5 mmHg/(ml/min · 100 g). This quite pronounced effect in skeletal muscle, one of the main targets for vasomotor control concerned with the maintenance of general cardiovascular homeostasis, suggest that β-adrenergic inhibition of vascular tone must be taken into account in studies aimed to investigate the control of the resistance function in bleeding, a research field which currently receives great interest. The inhibition of precapillary sphincter tone during hypovolemia has been attributed to the action of "vasodilator metabolites" accumulated as a result of the reduced blood flow (*e.g.* Lewis and Mellander 1962, Lundgren, Lundvall and Mellander 1964, Öberg 1964). The present findings

indicate that this response, which might be considered an important compensatory mech ism facilitating capillary exchange, instead might be mainly mediated by β-adrenocep activation at least during bleeding of short duration. It is possible, however that the rela importance of "vasodilator metabolites" for the precapillary sphincter dilatation will greater at more severe hemorrhage due to the attendant, marked decrease of regional bl supply Furthermore, vascular transmural pressure in the sphincter section may decre significantly during pronounced hemorrhagic hypotension and cause a myogenic inhibit of precapillary sphincter tone (cf Cobbold et al 1963). Yet, preliminary data from an tended series of experiments in which graded bleeding was performed indicate that the adrenergic mechanism is mainly responsible for the inhibition of precapillary sphincter t also during severe hemorrhage, and, further that the β-adrenergic dilator influence m resistance vessels during bleeding can be even more pronounced than in the present stu

The β-adrenergic dilator influence in the muscle vascular bed during hemorrhage may both neurogenic and humoral in origin. Sympathetic activation of the skeletal muscle vasc bed has been shown to produce a neurogenic β-adrenergic dilator response markedly aff ing the precapillary sphincters and the blood flow distribution in the muscle (Vyeros, C lick and Renkin 1968 Lundvall and Järhult 1974 1976). This neurogenic β-dilator mech ism seems, however of little importance for the volume flow of blood as regulated by t muscle vascular resistance (Lundvall and Järhult 1976). The observed β-adrenergic inhibi of vascular tone in the resistance vessels may instead by mainly related to the greatly creased release of adrenaline from the suprarenal glands during bleeding (e.g Watts 19 Hall and Hodge 1971 Jakschik et al 1974), an assumption supported by some prelimin experiments in which hemorrhage was performed before and after removal of the adren The blood-borne cathecolamines may also contribute to the dilation of the precapill sphincters during bleeding as suggested by infusion experiments (Lundvall and Hillman be published) — The question may be raised in this context whether the observed β-adr ergic dilator effects in the resistance vessels and precapillary sphincters during hemorrh reflect direct interaction with the specific receptors of the vascular smooth muscle or if t are indirect and secondary to alterations of the skeletal muscle metabolism produced by adrenergic substances. This problem, the site of action of catecholamines in vasodilatati has since long been a matter of debate and the present results do not permit any conclusi to be drawn With regard to skeletal muscle, however current views seem to strongly fav the mechanism of direct stimulation of β-receptors of the vascular effector cells. The recen described β-adrenergic facilitation of transmittor release from the adrenergic nerve endi (cf Langer 1977) also deserves consideration when discussing the site of action of the adrenoceptor mediated inhibition of vascular tone during hemorrhage. Blockade of any su presynaptic β-effect would, in fact, tend to diminish the recorded increase of vascular tone the resistance vessels and precapillary sphincters after administration of propranolol, there leading to an underestimation of the postsynaptic β-adrenergic dilator influence.

The most prominent alteration after β-blockade of the vascular response to bleeding w the pronounced reduction of the net transcapillary fluid absorption. Two main mechanis have been proposed to be responsible for the important, compensatory fluid transfer fro ... to the intravascular compartment during hypovolemia. The hemorrhage induc

increase of the sympatho-adrenal activity elicits a resetting of the pre to postcapillary resistance ratio leading to decreased capillary hydrostatic pressure and thereby to fluid absorption (Öberg 1964) and arterial hyperosmolality due to glucose release from the liver causes an osmotic fluid absorption (Järhult *et al.* 1972, Järhult 1973). The observed reduction of the fluid transfer in response to β-blockade would *a priori* seem related to an interference of the blocking agent with the adrenergically evoked fluid transfer an assumption supported by the fact that the osmolar changes in the blood during the present relatively mild and short-term bleeding was small and quite similar before and after β-blockade. The contribution of osmotic fluid transfer to the observed total fluid absorption observed may in fact be estimated from the measured osmolar changes and blood flow Thus, a previous experimental study (Lundvall 1972) showed that non-protein osmotic fluid transfer in the cat lower leg muscle, being flow dependent, is directly proportional to the osmolar uptake in the muscle, an experimental finding supported by theoretical analyses (Lundvall 1972, Salathe and An 1976). Experiments in which hypertonic glucose was administered i.v. so as to produce an arterial hyperglycemia and hyperosmolality in the range encountered in bleeding, i.e. 0–30 mOsm/kg H_2O (*cf.* Järhult *et al.* 1976), confirm the mentioned previous observations and show that the glucose osmotic fluid flux (ml/min 100 g) in cat muscle can be calculated from the following numerical product volume flow of blood (in ml/min 100 g) the arterio-venous osmolar difference (in mOsm/kg H_2O) 1 75 10^{-3} (Lundvall, unpublished *cf.* also Lundvall 1972) Using this formula, the average rate of osmotic fluid transfer at the time of final sampling for osmolality determination in the present hemorrhage expts. would amount to [illegible] ml/min 100 g in the intact muscle region and to 0.012 ml/min 100 g in the β-blocked region. Subtraction of these values from the observed rates of fluid transfer during bleeding indicates that, in the intact region, net transcapillary fluid absorption due to decreased capillary hydrostatic pressure amounted to 0.049 ml/min 100 g, corresponding to [illegible] of the total fluid gain, and that it was negligible in the β-blocked region. These deductions indicate that the fluid transfer from skeletal muscle to the blood stream in the present hemorrhage expts. mainly was due to reflex adrenergic mechanisms and that β-adrenoceptor activation was essential for the adrenergically evoked fluid gain.

It seems likely that the β-adrenergic mediation of fluid transfer from muscle to blood during hemorrhage is causally linked to the observed β-dilator vascular effects and that it explains the neurogenic and humoral β-adrenergic facilitation of hydrodynamic transcapillary exchange in skeletal muscle that can be observed during sympathetic stimulation and during infusion of adrenaline and noradrenaline (Lundvall and Järhult 1976, Lundvall and Hillman, to be published). The β-adrenergic inhibition of precapillary sphincter tone, leading to an increased number of patent capillaries and, hence, to an increased capillary surface area available for transcapillary fluid exchange, must have contributed significantly to the observed, pronounced β-adrenergic facilitation of the fluid transfer in bleeding. It can be deduced, however that a β-adrenergic adjustment of the pre to postcapillary resistance ratio leading to a fall of capillary hydrostatic pressure, must have been at least equally important as the precapillary sphincter dilatation. Such a resetting of the pre to postcapillary resistance ratio indicates that the β-adrenergic dilator influence, in *relative* terms, was more pronounced in the postcapillary than in the precapillary resistance vessels.

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Vasodilatation and modulation of vasoconstriction in canine subcutaneous adipose tissue caused by activation of β-adrenoceptors

By

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Abstract

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The present experiments were undertaken to study the balance between vascular α- and β-adrenoceptors in canine subcutaneous adipose tissue during sympathetic nerve stimulation and noradrenaline injections. Propranolol potentiated and prolonged the vasoconstrictor response to close i.a. injections of noradrenaline. The vasoconstriction induced by brief nerve stimulation (0.5 to 8 Hz) was, however, unaltered by the β-adrenoceptor blockade. During prolonged nerve stimulation the vasoconstrictor response was well maintained at 1.5 Hz but at 4 Hz there was a gradual escape. The escape phenomenon at 4 Hz was diminished by propranolol. The β_1-selective antagonist practolol, like propranolol, potentiated and prolonged the vasoconstriction induced by noradrenaline injections and reduced the vasoconstrictor escape during prolonged nerve stimulation at 4 Hz. Furthermore, the vasodilatation induced by noradrenaline injection or nerve stimulation during α-adrenoceptor blockade was diminished by practolol. Practolol also blocked the lipolytic response to noradrenaline and nerve stimulation. The β_2-selective antagonist H35/25 blocked the effects of the β_2-selective agonist salbutamol but failed to alter noradrenaline as well as nerve stimulation induced vascular and lipolytic β-adrenoceptor responses. The present results provide further support for the hypothesis that vascular β-adrenoceptors in adipose tissue are humoral (noninnervated), preferentially activated by circulating noradrenaline. Moreover, both vascular and lipolytic β-adrenoceptors activated by noradrenaline in adipose tissue are best classified as β_1-adrenoceptors.

The lipolytic response to intravascularly administered noradrenaline as well as that of noradrenaline released from the adrenergic nerve terminals in canine subcutaneous adipose tissue is mediated via activation of β-adrenoceptors (Fredholm and Rosell 1968, Ballard *et al.* 1971). The vascular responses to those stimuli are however qualitatively different. Thus, noradrenaline released from nerve terminals invariably causes α-adrenoceptor mediated vasoconstriction (Ngai *et al.* 1966, Hoffbrand and Forsyth 1973) whereas intravascular noradrenaline may induce vasodilatation (Nielsen *et al.* 1968, Ballard *et al.* 1971, Hoffbrand and Forsyth 1973). The explanation for these differences in vascular responses may be that the vascular α-adrenoceptors are innervated receptors in close contact with the sympathetic nerve endings whereas the vascular β-adrenoceptors are humoral (noninner-

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recently been shown to be highly selective for β_1- and β_2-adrenoceptors respectively (Levy and Wilkenfeld 1969). In each experiment a control treatment with the chosen stimulus—i.e. sympathetic nerve stimulation, noradrenaline infusion i.a. or salbutamol infusion i.a.—was first applied and blood flow and glycerol release were followed. Either practolol or H35/25 was then administered and the same stimulus was applied after a period of 30–40 min to permit distribution of the antagonist. In some experiments, the preparation was then used to study the other antagonist in the same manner.

Sympathetic nerve stimulation was performed either for 30 min at 4 Hz or, after α-adrenoceptor blockade with hydergine (200 μg i.a.), for 4 min at 2 Hz. Noradrenaline was injected close i.a. (2×10^{-10}–10^{-9} mol). After hydergine treatment noradrenaline was infused at 2.3×10^{-10} mol per min in 6 min giving a calculated blood concentration of 4.1 to 10×10^{-9} M. Salbutamol was infused at 1.4 to 3.8×10^{-10} mol per min in 4-9 min giving a calculated blood concentration of 1.2 to 3.4×10^{-8} M. When these infusions of noradrenaline or salbutamol were performed the amount of agonist per infusion were the same throughout each experiment. Since the blood flow declined gradually in most experiments the blood concentrations of noradrenaline and salbutamol were usually highest during the latest infusion.

Evaluation of data

Vasodilatation was calculated from the per cent decrease in resistance (mmHg × 100 g × min × ml^{-1}) and vasoconstriction from the per cent decrease in conductance (ml × min^{-1} × 100 g^{-1} × $mmHg^{-1}$). Thus, both vasoconstrictions and vasodilatations are expressed in percentages between 0 and 100. Lipolysis were calculated as the net release of glycerol (μmol × 100 g^{-1}) by subtracting the basal glycerol release from the total amount released during and after injection of a drug or nerve stimulation. Statistical evaluation of the results was performed with Student's t-test for paired observations. The following drugs were used: Noradrenaline (l-arterenol HCl, SIGMA), Salbutamol (Salbutamol Sulphate, Allen and Hanbury), Practolol (Eraldin, ICI), H35/25 Hässle, Propranolol (Inderal, ICI), Hydergine® (Sandoz).

Results

Effect of β-adrenoceptor blockade on vasoconstriction

The vasoconstriction in adipose tissue induced by sympathetic nerve stimulation at 2 Hz was similar on both the control and the propranolol-treated sides, but the vasoconstriction induced by noradrenaline (10^{-9} mol i.a.) was increased and prolonged by propranolol pretreatment (Fig. 1). The reactive hyperaemia after the nerve stimulation period was greater on the control side than on the treated side. Isoprenaline (10^{-10} mol i.a.) caused vasodilatation only on the control side demonstrating a high degree of β-adrenoceptor blockade on the propranolol treated side.

The dose effect relationships from all experiments are shown in Fig. 2. The peak vasoconstriction induced by noradrenaline was increased in the propranolol treated side in comparison to control. No difference in vasoconstriction response between the two sides was however observed following sympathetic nerve stimulation. These results indicate that the vascular response during a 4 min nerve stimulation is due to activation of α-adrenoceptors only whereas intraarterial noradrenaline activates both α- and β-adrenoceptors concomitantly.

Effect of β-adrenoceptor blockade on vasoconstriction produced by prolonged nerve stimulations

Although there was no apparent activation of vascular β-adrenoceptors during nerve stimulation for 4 min the possibility of β-adrenoceptor activation during longer stimulation periods had to be considered. The vasoconstriction induced by sympathetic nerve stimulation at 13 Hz was sustained during the whole 30 min period of stimulation and of similar size both

vated) receptors preferentially accessible to circulating noradrenaline (Rosell and Belfrage 1975 Belfrage and Rosell 1976)

After α-adrenoceptor blockade, sympathetic nerve stimulation causes vasodilatation by stimulation of unmasked β-adrenoceptors (Ngai *et al.* 1966). It is likely that stimulation of these β-adrenoceptors reduces or modifies the vasoconstrictor response to sympathetic nerve activity in the absence of α-adrenoceptor blockade. This has, however not yet been experimentally verified and the first aim of this investigation was to study the balance tween vascular α and β-adrenoceptors, both during sympathetic nerve stimulation and during intraarterial administration of noradrenaline.

Most vascular β-adrenoceptors have been suggested to be of the β_2-type (Lands *et al.* 1967) and noradrenaline, being predominantly a β -agonist, would thus be expected to have weak actions on vascular β-adrenoceptors However the β-adrenoceptors mediating vasodilatation in adipose tissue seems to be more sensitive to noradrenaline than those of other tissues (Hofbrand and Forsyth 1973 Hjemdahl and Fredholm 1976). Thus, vascular β-adrenoceptors in adipose tissue may be of the β rather than the β_2-type. The second aim of this investigation was to examine closely the classification of both vascular and lipolytic β-adrenoceptors in adipose tissue, according to the β_1 and β_2-adrenoceptor concept.

Methods

The experiments were performed on female mongrel dogs weighing between 8–20 kg. The dogs were anesthetized with sodium pentobarbital (25–30 mg/kg with supplements when necessary). All dogs were given heparin (2 500 units v./kg kindly supplied by AB Vitrum) to prevent blood clotting. The dogs were mechanically ventilated through tracheotomy The inguinal subcutaneous adipose tissue on both the right and the left side were isolated and autoperfused with blood from a femoral artery (Rosell 1966). Blood flow was measured with an arterial drop recorder and registered together with the blood pressure recorded from carotid artery on 7B Grass Polygraph. The vein draining the adipose tissue was also canulated in some experiments and a three way stop cook permitted sampling of venous blood for glycerol analyzes (Laurell and Tibbling 1966). The adipose tissue nerve, containing sympathetic fibers, was sectioned in all experiments. In those cases where the effects of sympathetic nerve stimulations were studied, the nerve was placed on a bipolar electrode and stimulated at supramaximal intensity (15V) and duration (2 ms).

Effect of β-adrenoceptor blockade on vasoconstriction induced by sympathetic nerve stimulation and intra-arterial noradrenaline injections

The adipose tissue on one side was initially treated with 500 μg propranolol i.a and 100–200 μg were repeatedly given to assure a complete β-adrenoceptor blockade. The venous blood was collected for 3–5 min after each injectio and discarded to minimize the systemic dose of propranolol. Both sides were simultaneously stimulated at the same frequency or treated with the same dose of noradrenaline and the untreated adipose tissue preparation served as control. The parameter studied was the peak vasoconstriction induced by sympathetic nerve stimulation (0.5–8 Hz) for 4 min or by close intra arterial injections of noradrenaline (10^{-10} 10^{-8} mol). Data for frequency and dose response curves were btained from 5 dogs.

Effect of β-blockade on prolonged (30 minutes) sympathetic nerve stimulations

In 4 dogs one adipose tissue preparation was stimulated at 1.5 Hz for 30 mi and the other simultaneously stimulated at 4 Hz for the same period of time. 500 μg propranolol i.a. was then dministered to both sides and the stimulations repeated. Blood flows and glycerol release were followed.

Effect of practolol and H35/25 on the vascular and lipolytic effects of nerve stimulation, salbutamol and noradrenaline

In these studies practolol 1 mg/kg i.v was used as β_1-adrenoceptor blocking substance and H35/25 2 mg/kg i.v as a β_2-adrenoceptor blocking substance. Practolol and H35

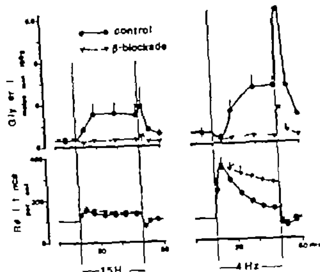


Fig. 1. Time course of vasoconstriction and glycerol release during and following sympathetic nerve stimulation for 30 min. The figures are the mean ± S.E. from 4 dogs. Black dot ( ) indicates significant difference between pre- and post-treatment (β-blockade) and control.

...ctive antagonist practolol (1 mg/kg i.v.) both the vasodilator and the lipolytic response ... butamol ... ere unaltered. However practolol inhibited lipolysis as well as the vaso... ...iction escape during the nerve stimulation. When the treatments were repeated a ... time after administration of the β_1-selective antagonist H35/25 (2 mg/kg i.v.) there ...o further alteration of the effects to sympathetic nerve stimulation whereas both ...tion and lipolysis induced by salbutamol were inhibited. This pattern of agonist-...onist interaction was found in all experiments both for vasodilatation and lipolysis. ... the vasodilator component in the response to i.a. injection of noradrenaline as well as

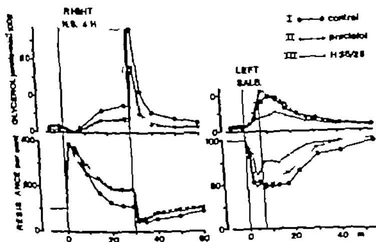


Fig. 4. Time course of the vascular and lipolytic effects of sympathetic nerve stimulation (for 30 min at 4 Hz) to the right side and of i.a. infusion of salbutamol (2.0 10^{-9} mol during 7 min) to the left side subcutaneous adipose tissue in one dog. Treatments were induced three times consecutively. I control II 30 min after administration of practolol (1 mg/kg i.) and III 30 min after administration of H35/25 (2 mg/kg i.).

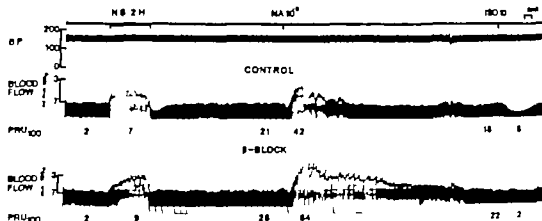


Fig. 1 Effect of propranolol on vascular responses to sympathetic nerve stimulation for 4 min at 2 Hz (N.S.) i.a. injection of noradrenaline 10^{-9} mol (NA) and i.a. injection of isoprenaline (ISO), in two simultaneously perfused adipose tissue preparations in one expt. Recordings are as follows from the top: time scale, systemic blood pressure (BP) and blood flow on the control and the propranolol treated (β-block) side. Values for calculated resistance in PRU_{100} (BP/Blood flow) are given at intervals below each blood flow tracing.

before and after propranolol treatment (Fig. 3) Stimulation at 4 Hz caused the same peak vasoconstriction on both sides but on the control side the response gradually diminished during the 30 min-period of stimulation This escape was reduced on the propranolol treated side. The lipolysis induced by the 1.5 Hz stimulation was almost the same as that induced by 4 Hz. Propranolol completely inhibited lipolysis induced by both frequencies.

The effect of β and $β_2$-selective antagonists

Fig. 4 shows results of an expt. in which salbutamol (2.0 10^{-8} moles) was infused to the left adipose tissue preparation for 7 min and where the nerve to the right side tissue was stimulated for 30 min at 4 Hz. When these treatments were repeated after administration of the

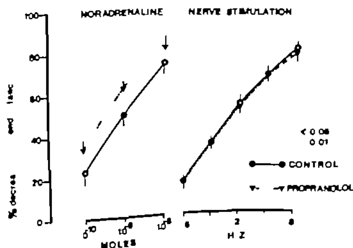


Fig. 2. Effect of β-adrenoceptor blockade with propranolol on peak vasoconstriction to noradrenaline and to sympathetic nerve stimulation for 4 min. Figures are given as mean ± S.E. from 5 dogs.

Table III Effect of β-adrenoceptor blockade on lipolysis in 9 dogs. The net release of glycerol after ranged treatments are given. When both β-blockers were used H35/25 was administered prior to practolol except in expt. 5 as indicated. SALB = i.a. infusion of salbutamol, N.S. = sympathetic nerve stimulation, NA = i.a. injection or infusion of noradrenaline.

Expt. no	Treatment	Net glycerol release (μmol 100 g⁻¹)		
		Control	H35/25	Practolol
1	SALB	42.8	—	35.9
2	SALB	5.2	0.3	3.8
3	SALB	70.7	15.4	37.4
	N.S.	62.5	50.2	10.8
4	SALB	13.3	2.2	6.2
	N.S.	26.8	17.1	0.4
5	SALB	69.9	33.0*	68.6
	N.S.	116.0	31.5	27.6
6	N.S.	79.6	—	23.7
	N.S.	53.0	—	9.9
	NA	17.4	—	3.3
	NA	2.9	—	0
7	NA	5.5	—	0.5
	NA	40.2	—	14.9
8	NA (α-blockade)	3.8	—	1.2
	N.S. (α-blockade)	2.0	—	0.2
9	NA (α-blockade)	7.5	9.8	0
	N.S. (α-blockade)	9.8	12.2	1.8

* Indicates that the practolol period preceded the H35/25 period.

and Fredholm 1976). On the other hand vasoconstriction induced by sympathetic nerve stimulation in canine subcutaneous adipose tissue was unaffected by propranolol. The presence of presynaptic β-adrenoceptors facilitating the release of noradrenaline from the sympathetic nerve terminals (see Langer 1977) may offer an explanation for these differences since any increased vasoconstrictor response due to blockade of the vascular β-adrenoceptors may be masked by the decreased noradrenaline release caused by a blockade of presynaptic β-adrenoceptors. It seems unlikely however that these two different actions of the β-blockade should exactly balance each other at all frequencies tested (0.5–8 Hz), especially since the presynaptic β-adrenoceptors seems to be of importance mainly at very low frequencies (see Langer 1977). A more likely explanation for the unaltered vasoconstrictor response to sympathetic nerve stimulation during β-adrenoceptor blockade is that noradrenaline released from nerve terminals cause α-adrenoceptor activation only. This would explain why sympathetic nerve stimulation invariably causes vasoconstriction in subcutaneous adipose tissue (Ngai *et al.* 1966). The present results are thus in good agreement with the hypothesis that vascular β-adrenoceptors in adipose tissue are humoral (non-innervated) receptors whereas the vascular α-adrenoceptors are innervated receptors (Rosell and Belfrage 1975, Belfrage and Rosell 1976).

During brief nerve stimulation there was little evidence for activation of vascular β-

TABLE I Effect of β-adrenoceptor blockade on a) escape of vasoconstriction during nerve stimulation at 4 Hz and b) peak vasoconstriction to i.a. injection of noradrenaline. The figures are given as mean ± S.E. when n > 3

	Vasoconstriction % decrease in conductance		N	β-blocker
	Control	β-blockade		
a) Vasoconstriction at end of nerve stimulation for 30 min at 4 Hz	48±8	62±4	4	Propranolol
	25±7	47±5	3	Practolol
	4 and 34	31 and 26		H35/25
b) Peak vasoconstriction to noradrenaline 10^{-8} mol i.a.	50±5	62±4	5	Propranolol
	54±3	62±2	4	Practolol

Significant ($p < 0.05$) difference from corresponding control.

the vasoconstrictor escape during 30 min nerve stimulations at 4 Hz was only inhibited b[y] practolol (Table I). Practolol but not 35/25 diminished the vasodilating effects of noradren[aline] and nerve stimulation during α-adrenoceptor blockade whereas H35/25 but no[t] practolol diminished the vasodilator response to salbutamol (Table II) Table III shows tha[t] practolol blocked the lipolytic responses to nerve stimulation and to noradrenaline to [a] greater extent than H35/25 However H35/25 was much more effective than practolol i[n] blocking the lipolytic responses to salbutamol

Discussion

In the present study it was confirmed that β-adrenoceptor blockade with propranolo[l] potentiates the vasoconstriction induced by close i.a. injection of noradrenaline (Hjemdah[l]

TABLE II Effect of β-adrenoceptor blockade on vasodilatation induced by a) sympathetic nerve stimulatio[n] after α-adrenoceptor blockade b) i.a. infusion of noradrenaline after α-adrenoceptor blockad[e] and c) intraarterial infusion of salbutamol. The figures are then mean ± S.E. changes durin[g] the last minute of infusion or nerve stimulation

		Concentration of SALB and NA	Vasodilatation % decrease in resistance	
a) Nerve stimulation α-blockade	Control	—	47±9	3
	Practolol	—	28±11	
	Control	—	29/42	2
	H35/25	—	36/38	
b) NA-infusion α-blockade	Co trol	7.2±1 6 10^{-8} M	51±9	3
	Practolol	7.8±1 4 10^{-8} M	29±12	
	Control	4 1/8.2 10^{-8} M	39/45	2
	H35/25	6.2/9 8 10^{-8} M	42/51	
c) SALB-infusion	Control	1.3/1 4 10⁻ M	47/53	2
	Practolol	1 6/2.1 10^{-7} M	52/63	
	Control	1 6±0.2 10⁻ M	46±3	3
	H35/25	1 8±0.2 10^{-7} M	37±2*	

Significant ($p < 0.05$) difference from corresponding control.

TABLE III Effect of β-adrenoceptor blockade on lipolysis in 9 dogs. The net release of glycerol after staged treatments are given. When both β-blockers were used H35/25 was administered prior to practolol except in expt. 5 as indicated. SALB = i.a. infusion of salbutamol, N.S. = sympathetic nerve stimulation, NA = i.a. injection or infusion of noradrenaline.

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and Fredholm 1976). On the other hand vasoconstriction induced by sympathetic nerve stimulation in canine subcutaneous adipose tissue was unaffected by propranolol. The presence of presynaptic β-adrenoceptors facilitating the release of noradrenaline from the sympathetic nerve terminals (see Langer 1977) may offer an explanation for these differences since any increased vasoconstrictor response due to blockade of the vascular β-adrenoceptors may be masked by the decreased noradrenaline release caused by a blockade of presynaptic β-adrenoceptors. It seems unlikely however that these two different actions of the β-blockade should exactly balance each other at all frequencies tested (0.5–8 Hz), especially since the presynaptic β-adrenoceptors seems to be of importance mainly at very low frequencies (see Langer 1977). A more likely explanation for the unaltered vasoconstrictor response to sympathetic nerve stimulation during β-adrenoceptor blockade is that noradrenaline released from nerve terminals cause α-adrenoceptor activation only. This would [illegible] by sympathetic nerve stimulation invariably causes vasoconstriction in subcutaneous adipose tissue (Ngai *et al.* 1966). The present results are thus in good agreement with the hypothesis that vascular β-adrenoceptors in adipose tissue are humoral (non-innervated) receptors whereas the vascular α-adrenoceptors are innervated receptors (Rosell and Belfrage 1975, Belfrage and Rosell 1976).

During brief nerve stimulation there was little evidence for activation of vascular β-

adrenoceptors. However during prolonged nerve stimulation at 4 Hz there was a vaso-constrictor escape which could be diminished by propranolol There are several alternatives to explain the mechanism behind this β-adrenoceptor mediated phenomenon. Firstly the slow onset of the escape might suggest that it is secondary to increased metabolism in the tissue. However there does not seem to be a simple relationship between the escape and lipolysis since the lipolysis at 1 5 Hz was almost as large as at 4 Hz. The combination of increased metabolism and a high degree of vasoconstriction may on the other hand be of importance since this may lead to accumulation of vasodilating products such as adenosine (Fredholm 1976) which may contribute to the escape phenomenon (Fredholm and Sollevi 1977) Secondly at higher frequencies, there might be a gradual overflow of noradrenaline from the nerve terminals at the adventitio-medial border to the humoral (noninnervated) β-adrenoceptors in the media of the blood vessels. At a lower frequency such as 1.5 Hz, the amount of noradrenaline reaching the vascular β-adrenoceptors could be too small to cause any significant activation of these receptors.

In the present study it was confirmed that sympathetic nerve stimulation after α-adrenoceptor blockade, invariably causes β-adrenoceptor mediated vasodilatation in adipose tissue (Ngai *et al* 1966) A possible explanation for these differences in β-adrenoceptor activation during normal condition and during α-adrenoceptor blockade may be a higher availability of noradrenaline at β-adrenoceptors caused by the α-adrenoceptor blockade This could be caused in two ways. Firstly presynaptic α-adrenoceptor blockade may cause increased noradrenaline release from the sympathetic nerve endings (see Langer 1977) and possibly the amounts reaching the β-adrenoceptors will be higher than during normal conditions. Secondly the lack of vasoconstriction caused by the postsynaptic α-adrenoceptor blockade may increase the noradrenaline outflow contributing to increased noradrenaline concentration (Rosell, Kopin and Axelrod 1964). Indeed, it has been found that α-adrenoceptor blockade increases the amounts of noradrenaline escaping to the venous effluent following sympathetic nerve stimulation in subcutaneous adipose tissue (Fredholm and Rosell 1970) Thus, although sympathetic nerve stimulation causes β-adrenoceptor mediated vasodilatation in adipose tissue during α-adrenoceptor blockade the present study suggests that during normal conditions there is no activation of vascular β-adrenoceptors of quantitative importance during sympathetic nerve stimulation, the vasoconstrictor escape being one possible exception

In addition to a direct activation of vascular β-adrenoceptors catecholamines may also induce vasodilatation secondary to an increased metabolism in the surrounding tissue (see Lundholm 1966). In the present study it was found that the β -selective antagonist practolol blocked both lipolysis as well as four different manifestation of β-adrenoceptor mediated vasodilatation induced by noradrenaline or sympathetic nerve stimulation. These findings may support the view that the noradrenaline induced vasodilatation is secondary to lipolysis (Nielsen *et al* 1968) However there does not seem to be a causal relationship between those two parameters. Thus, nicotinic acid blocked lipolysis but not vasodilatation induced by i.v noradrenaline (Mjös and Akre 1971) Blockade of neuronal uptake of noradrenaline potentiated lipolysis but did not alter vasodilatation induced by i.a. injection of noradrenaline during α-adrenoceptor blockade (Rosell and Belfrage 1975 Belfrage and Rosell 1976).

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…present findings that practolol blocks both lipolysis and vasodilatation induced by …enaline may suggest that in adipose tissue both the β-adrenoceptors on the fat cells …e vascular β-adrenoceptors are of the β_1-type. However in the present study both …tation and lipolysis induced by salbutamol were more effectively blocked by the …ctive antagonist H35/25 than by practolol. Thus, both the vasodilating and the …ic responses to β_2-selective agonists in adipose tissue are preferentially blocked by …ctive antagonists whereas the same responses to β_1-selective agonists are preferentially …d by β_1-selective antagonists. Carlsson *et al.* (1972) reported the same pattern of …t-antagonist interactions in the cat heart and suggested that both β_1- and β_2-adreno…s are present in this tissue exerting the same response. Thus, both β_1- and β_2-adreno…s may be present both in the resistance vessels and on the fat cells in adipose tissue. …er other factors such as the local distribution of these drugs in the tissue may also …importance for the agonist–antagonist interaction patterns reported here for adipose …

…onclusion the present study suggests that two factors may be of major importance …e vasodilating properties of intravascular noradrenaline in adipose tissue. Firstly …scular β-adrenoceptors seem to be humoral (noninnervated) preferentially accessible …ulating noradrenaline. Neuronally released noradrenaline may only during special …stances be able to activate those receptors. Secondly the vascular β-adrenoceptors …ed by noradrenaline seems mainly to be of the β_1-type. Since vascular β-adreno…s in … skeletal muscle are of the β_2-type (Lands *et al.* 1967) the presence of β_1-…ceptor in adipose tissue could offer one explanation why adipose tissue was the only … that responded with vasodilatation to intravenous infusion of noradrenaline (Hoff… and Forsyth 1973).

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Comparison of β-adrenoceptors mediating vasodilatation in canine subcutaneous adipose tissue and skeletal muscle

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Abstract

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Blood flow changes in response to various drugs in simultaneously autoperfused canine subcutaneous adipose tissue and gracilis muscle were compared to study the vascular β-adrenoceptors. Compared to isoprenaline the β_2-selective agonist salbutamol was 4–6 times more potent as vasodilator in the muscle than in adipose tissue. Furthermore two β_1-selective agonists (Tazolol and H80/62) caused vasodilatation in adipose tissue but not in the gracilis muscle. When given by close i.a. injection after β-adrenoceptor blockade, adrenaline was a more potent vasoconstrictor than noradrenaline in both tissues. Before β-blockade, however, noradrenaline was the more potent vasoconstrictor in the gracilis muscle whereas adrenaline was more potent in adipose tissue. Intravenous infusion of adrenaline in doses causing vasodilatation in the muscle caused vasoconstriction in adipose tissue whereas intravenous infusion of noradrenaline caused vasoconstriction in both tissues. The present findings suggest that the β-adrenoceptors mediating vasodilatation in skeletal muscle are mainly of the β_2-type, whereas β_1-adrenoceptors seem to predominate in subcutaneous adipose tissue. Since adrenaline is a much more potent β_2- than β_1-agonist, these differences point to different roles of intravascular adrenaline in the two sites. In skeletal muscle circulating adrenaline is mainly a vasodilator whereas in subcutaneous adipose tissue it mainly acts as a vasoconstrictor.

In contrast to other tissues (Hoffbrand and Forsyth 1973) subcutaneous adipose tissue may respond with β-adrenoceptor mediated vasodilatation during intravascular administration of noradrenaline (Nielsen *et al.* 1968, Ballard *et al.* 1971, Hoffbrand and Forsyth 1973). Sympathetic nerve stimulation, on the other hand, leads mainly to α-adrenoceptor activation (Belfrage 1978) and thus always causes vasoconstriction in adipose tissue (Ngai *et al.* 1966) as in other tissues. The vasodilating properties of intravascular noradrenaline in adipose tissue may have several explanations. Firstly vascular α- and β-adrenoceptors may have different distribution in relation to the sympathetic nerves—the α-adrenoceptors being innervated receptors in close contact with nerve endings and the β-adrenoceptors being humoral (noninnervated) receptors situated closer to the lumen at some distance from the nerve terminals (Rosell and Belfrage 1975, Belfrage and Rosell 1976). Such an arrangement

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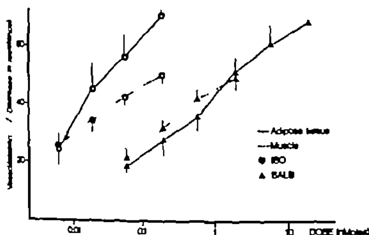
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ig. 1 Dose-response curves to l isoprenaline (ISO) and salbutamol (SALB) in adipose tissue and in the acilis muscle. Each symbol represents the mean ± S.E. in four dogs. Salbutamol 20 nmol was tested in e dog only

Results

he resting blood flows in the two tissues were calculated in 11 dogs and found to be 15.9 l min⁻¹ 100 g tissue⁻¹ (range 6.2–26.2) in adipose tissue and 14.6 ml min⁻¹ 100 g ssue⁻¹ (12.0–16.5) in the gracilis muscle. Thus resting blood flows were similar in the two ues although there was greater variation in adipose tissue.

In four dogs the vasodilating effects of isoprenaline and salbutamol were compared Fig. 1). In the gracilis muscle the dose-response curves for the two compounds were parallel d salbutamol was about 12 16 times less potent than isoprenaline. The dose-ratio at the sponse level of 40% decrease in resistance was 14 ± 1 1 In adipose tissue, on the other nd the dose-response curve for isoprenaline was somewhat steeper than for salbutamol aking dose-ratio calculations dependent on the size of the response. At 40 and 55% decrease in resistance the dose-ratios were 52 ± 8 and 93 ± 9 respectively Both these two se-ratios are significantly ($p < 0.05$) different from the dose-ratio in the skeletal muscle. hus, in relation to isoprenaline, which is an agonist on both β_1- and β_2-adrenoceptors, the selective agonist salbutamol was some 4–6 times less potent a vasodilator in adipose ue compared with the gracilis muscle.

The two β_1-selective agonists tazolol (ITP) and H 80/62 induced dose dependent vasodilations in adipose tissue (Fig. 2). Although these drugs were not always tested in the me preparations as isoprenaline it could be calculated that they were about 500 times less tent than isoprenaline by comparing the dose response curves of Fig. 1 and Fig. 2. Both zolol and H 80/62 failed to alter the blood flow in the gracilis muscle. The vasodilatations adipose tissue induced by ITP and H 80/62 could be blocked by pretreatment with propranolol 500 µg i.a.

When given by close i.a. injection both noradrenaline and adrenaline caused dose dependent vasoconstriction in adipose tissue as well as in the gracilis muscle (Fig. 3). In control ans adrenaline was the more potent vasoconstrictor in adipose tissue whereas noradren-

of vascular α and β-adrenoceptors has earlier been proposed for other tissues e.g. skel muscle (Glick et al 1967) An additional explanation could be the existence of differ types of vascular β-adrenoceptors in different tissues. Hjemdahl and Fredholm (1976) s gested that adipose tissue in contrast to skeletal muscle may have a β-adrenergic vasodil mechanism that is unusually sensitive to noradrenaline. Since noradrenaline is a somew specific β -agonist and vascular β-adrenoceptors in general are of the β_2-type (Lands et 1967) a predominance of β -adrenoceptors in the resistance vessels of adipose tissue co help to explain the vasodilating properties of intravascular noradrenaline in this tis There is some experimental evidence that adipose tissue have vascular β-adrenoceptor the β type (Belfrage 1978) To provide further experimental evidence for the existenc different types of vascular β-receptors I have in the present study made a direct compari of vasodilatation mediated by β-adrenoceptors in s.c. adipose tissue and in skeletal mus

Methods

Female mongrel dogs weighing 10–22 kg were anesthetized with sodium pentobarbitone, 30 mg/kg supplemented as necessary Tracheotomy was performed and the dogs were mechanically ventilated

Subcutaneous adipose tissue weighing 43 g (range 28–55 g) in the inguinal region of 17 dogs was prep according to the method of Rosell (1966). The adipose tissue was completely isolated from all surroun tissues and supplied by one artery one vein and a single nerve which was sectioned in all expts. The co laterial gracilis muscle weighi g 55 g (range 30–80 g) was completely isolated from surrounding tissu described by Renkin and Rosell (1962). This tissue was also denervated in all expts.

Heparin, 2 500 I.U./kg i.v was administered to prevent clotting and the adipose tissue artery and artery to the gracilis muscle were cannulated and autoperfused from the ipsilateral femoral artery adipose tissue vein was cannulated in all expts. and the blood was returned to the femoral vein. The gr muscle vein(s) were not cannulated. Silicon filled drop recorders for blood flow registration were ins in the arterial loops. Systemic blood pressure was measured in carotid artery and recorded together blood flow on a Grass model 7B polygraph. Vasodilatation was calculated from the per cent decrea resistance (mmHg 100 g min ml^{-}) and vasoconstriction from the per cent decrease om conduct (ml × min^{-1} 100 g^{-1} mmHg^{-1}). Thus, both vasoconstrictions nd vasodilatations are expressed in centages between 0 and 100. Drugs were given simultaneously to both tissues by close i.a. bolus injec through side-arms in the arterial loops, proximal to the drop recorder All drugs were freshly mad from frozen stock solutions and diluted in saline containing 20 μg/ml ascorbic acid. Injection volume always 0.2 ml. I jection of this volume f the saline-ascorbic acid solution had no vascular effects. In experiments, where adrenaline and noradrenaline were injected, propranolol (500 μg i.a.) was administ to each tissue after the co trol dose response curves were determined and a second pair of dose resp curves were then obtained. This dose of propranolol gives an almost complete blockade of β-adrenocep (Fredholm and Rosell 1968). In 5 of the expts. adrenaline and noradrenaline were infused i. throu brachial vein in different concentrations at rate of 0.23 ml/min for 10–20 min. Infusion of the sa ascorbic acid solution t this rate had no effect on blood pressure or the adipose tissue and gracilis m blood flows.

I 4 additional expts. two adipose tissue preparations were simultaneously perfused, one pretreated propranolol (500 μg i.a.) and the other serving as control. The vascular as well as the lipolytic respo to i.v infusion of noradrenaline and adrenaline was measured. The release of glycerol was taken a index of lipolytic activity and was measured according to the method of Laurell and T bbling (1 Venous blood samples were taken from the adipose tissue vein and arterial samples from the carotid arte The glycerol release rate was calculated by multiplying the A V difference by plasma flow in ml min 100 g^{-}

The following drugs were used noradrenaline (L-arterenol HCl, Sigma), adrenaline (L-epineph bitartrate, Sigma), isoprenaline (L-isoprenaline bitartrate) salbutamol (salbutamol sulphate, Allen Hanbury), ITP (tazolol-HCl, Syntex), H 80/62 (±H 133/22, Hässle), propranolol (Inderal® ICI).

Statistical evaluation was performed by using Student's t-test for paired observations.

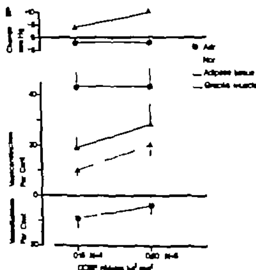


Fig. 4. Effect of i.v. infusion of adrenaline (Adr) and noradrenaline (Nor) in concomitantly perfused adipose tissue and gracilis muscle. The dose of 0.15 nmol Kg^{-1} min^{-1} was tested in 4 dogs, whereas the 4 times higher dose was tested in 5 dogs. Symbols represents the mean ± S.E. In adipose tissue adrenaline was significantly (p [illegible]) more potent vasoconstrictor than noradrenaline at both doses.

[illegible] was infused at a rate of 0.15 or 0.60 nmol kg^{-1} min^{-1} there was a fall in the mean systemic blood pressure in all expts., as well as a decreased vascular resistance in the gracilis muscle (Fig. 4). However in adipose tissue the concomitant vascular reaction was vasoconstriction. Infusion of noradrenaline in the same doses (0.15 and 0.60 nmol kg^{-1} min^{-1}) caused a small increase in systemic blood pressure and vasoconstriction in both

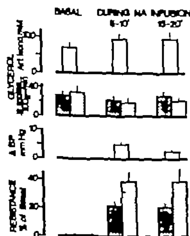


Fig. 5. Effect of i.v. infusion of 0.60 nmol kg^{-1} min^{-1} noradrenaline for 20 min on arterial glycerol concentration, glycerol release from two concomitantly perfused adipose tissue preparations, mean systemic blood pressure and vascular resistance in the adipose tissue preparations. One tissue was pretreated with the β-adrenoceptor blocking agent propranolol 500 µg i.a. (Dotted bars) and the other served as control (open bars). Mean values ± S.E. [illegible]

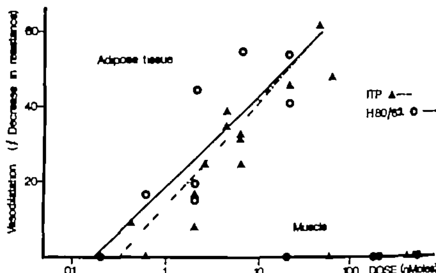


Fig. 2. Vasodilatation induced by ITP (Tazolol) and H 80/62. Each symbol represents the effect of an i.a. injection. ITP was tested in 5 adipose tissue and 3 gracilis muscle preparations and H 80/62 in 4 adipose tissue and 3 gracilis muscle preparations. Regression lines of the vasodilatations in adipose are also shown

aline was the more potent vasoconstrictor in the gracilis muscle. β-adrenoceptor blockade with propranolol (500 μg i.a.) did not alter the relationship between the two amines in adipose tissue. However after propranolol adrenaline became more potent as a vasoconstrictor than noradrenaline also in the gracilis muscle.

Intravenous infusions of adrenaline and noradrenaline were performed in 5 dogs. When

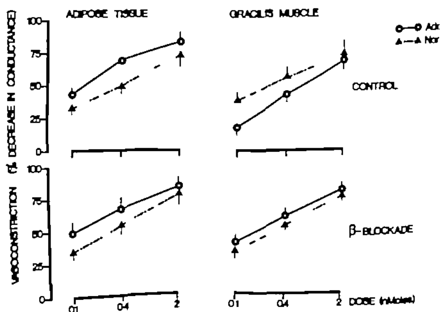


Fig. 3 Dose-response curves to i.a. adrenaline (Ad) and noradrenaline (Nor) in adipose tissue and in the gracilis muscle. Symbols represents the mean ± S.E. in 5 dogs. During control conditions (on top) adrenaline was significantly ($p < 0.01$) more potent than noradrenaline in adipose tissue whereas noradrenaline was significantly ($p < 0.01$) more potent in the muscle. After β-adrenoceptor blockade (bottom) adrenaline was the most potent vasoconstrictor in both tissues ($p < 0.05$).

of great importance for their vascular responses. In the present expts., adrenaline more potent vasoconstrictor than noradrenaline in both tissues after β-adrenoceptor ade. The present finding that adrenaline was a less potent vasoconstrictor than noradrenaline in the gracilis muscle but not in the adipose tissue, prior to β-blockade, could be explained by the predominance of adrenaline-sensitive β_2-receptors in the muscle, ing the α-adrenoceptors. During i.v. infusion of adrenaline the β_2-receptor effects dominating in the gracilis muscle resulting in vasodilatation in this tissue. However concomitantly perfused adipose tissue the vascular response was vasoconstriction. rmore, before β-blockade adrenaline was a more potent vasoconstrictor in adipose than noradrenaline both when the amines were given by close i.a. injection and when were infused i.v. Thus, the present study points to different roles of intravascular aline. In skeletal muscle it may mainly be a vasodilator whereas in adipose tissue it is constrictor.

e present study has shown marked differences in β-adrenoceptors mediating vasotion in adipose tissue and skeletal muscle. In the muscle only β_2-adrenoceptors seem present whereas β_1-adrenoceptors predominates in adipose tissue. The occurrence of the β_1-adrenoceptors has been proposed also in other tissues such as the kidney (Taira 1977) and the brain (Sercombe *et al.* 1977). The classification of vascular β-adrenoceptors into the β_2-type (Lands 1967) may thus be far too generalizing. The present study ts that the type of vascular β-adrenoceptor may be of great importance for the vascular nses to endogenous catecholamines. This is further supported by the difference in lar reaction in adipose tissue and in skeletal muscle to hemmorhagic shock in dogs g which blood flow in adipose is decreased to a much larger extent than in skeletal le (see Rosell *et al.* 1973). Since both tissues were denervated, and since α-adrenoceptor kade largely prevented the vasoconstriction it was suggested that increased levels of lating catecholamines caused the severe vasoconstriction in adipose tissue. In skeletal cle the α-adrenergic effects caused by increased blood levels of adrenaline may be teracted by an concomitant β_2-adrenoceptor activation. In adipose tissue, where vasr β_2-adrenoceptors is of minor importance the increased levels of adrenaline seems to e a severe, essentially unopposed vasoconstriction.

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adipose tissue and the gracilis muscle (Fig. 4). Compared to adrenaline, noradrenaline cause a much less pronounced vasoconstriction in adipose tissue. In adipose tissue the vaso constrictions induced by 0.15 and 0.60 nmol × kg⁻ min of both adrenaline and nor adrenaline were sustained throughout the 15–20 min infusion.

In 4 expts. 2 adipose tissue preparations were perfused, one of which was treated wit 500 μg propranolol. As can be seen in Fig. 5 intravenous infusion of 0.60 nmol kg^{-1} × min of noradrenaline caused a slight increase in systemic blood pressure and an increase in th arterial concentration of glycerol. However there was no increase in glycerol release from either the control preparation or that treated with propranolol. The vasoconstriction wa significantly greater ($p < 0.05$) in the β-blocked tissue both at 5–10 min and at 15–20 mi infusion.

Discussion

According to the classification of Lands *et al.* (1967) vascular β-adrenoceptors are of th β_2-type. This seems to hold for the resistance vessels of the gracilis muscle of the dogs, sinc the dose-ratio between the β_2-selective agonist salbutamol and isoprenaline in the presen study is in good agreement with what is usually found for such β_2-mediated effects as vaso dilatation in vascular beds of skeletal muscles (Cullum *et al.* 1969, Wasserman and Lev 1974, Taira *et al.* 1977) and bronchodilatation (Brittain *et al.* 1973, Cullum *et al.* 1969). In contrast, it has been suggested that the resistance vessels of dog subcutaneous adipos tissue contain mainly β -adrenoceptors, since a β but not a β_2-selective antagonist coul block the vasodilating effects of noradrenaline (Belfrage 1978). In the present study 4– times higher doses of salbutamol in relation to isoprenaline was needed in adipose tissue i comparison with the gracilis muscle. Furthermore tazolol and H 80/62 induced vasodilata tion in adipose tissue but not in the gracilis muscle. Both tazolol (Lockwood and Lum 1974 and H 80/62 (Hedberg *et al.* 1977) has been shown to have β -selective agonistic properties. Thus, the present study supports the view that vascular β-adrenoceptors in adipose tissu in contrast to skeletal muscle, are mainly of the β -type. However vascular β_2-adrenoceptor may also be present in adipose tissue since a β_2- but not a β -selective antagonist could bloc vasodilatation induced by salbutamol (Belfrage 1978).

These tissue differences in vascular β-adrenoceptor types may be of considerable im portance for the vascular responses to circulating adrenaline and noradrenaline since the catecholamines have different β and β_2-receptor agonist profiles. Noradrenaline is mainl a β -agonist (Lands *et al.* 1967). This may explain why adipose tissue in contrast to skelet muscle responded with vasodilatation to i.v. noradrenaline (Hoffbrand and Forsyth 1973. Vasodilatation in adipose tissue to i.v. infusion of noradrenaline was not seen in the presen study in contrast to others (Ballard 1973, Hjemdahl and Fredholm 1974). Possibly the differences may be explained by higher resting blood flows and lower doses of noradrenalin in the present study. However vascular β-adrenoceptor activation in adipose tissue coul be demonstrated also in this study since β-adrenoceptor blockade potentiated the vaso constriction induced by i.v. noradrenaline.

Adrenaline, in comparison with noradrenaline, is a much more potent β_2-agonist and weaker β_1-agonist (Lands 1967). The α-adrenoceptor agonistic properties of the two amines

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The *in vivo* transport of ^{14}C-α-aminoisobuturic acid into mouse blastocysts during activation for implantation

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Abstract

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[illegible] transport of ^{14}C-AIB (^{14}C-α-aminoisobuturic acid) into mouse blastocysts was studied during [illegible] for implantation. Mice, kept in experimentally delayed implantation, were given estrogen to [illegible] implantation and then injections of ^{14}C-AIB [illegible] at 0, 4 and 8 h after the estrogen. After *in vivo* [illegible] times for 1/3 or 4 h [illegible] the labeled amino acid the blastocysts were flushed out of the uterus [illegible]. A distinct uptake of ^{14}C-AIB occurred in the blastocysts 8 h (the highest uptake) and 12 h [illegible], provided that the in vivo incubation time was 4 h. At these times the blastocysts are [illegible] in the uterine lumen and consequently there is [illegible] transport of ^{14}C-AIB from the epithelium via [illegible] secretion into the blastocysts. This uptake indicates that amino acids transported by the system [illegible] important nutrients during early activation. The uptake and retention of ^{14}C-AIB in the uterus was [illegible] at 4 and 8 h after the induction of implantation. The highest uptake was observed [illegible] the labeled [illegible] was given at 8 h while the longest retention time occurred when ^{14}C-AIB was given at 4 h. [illegible] the transport ratio between the blastocysts and the uterine tissue is not [illegible] constant [illegible] concluded that the [illegible] rates for ^{14}C AIB transport are different for the uterus and the [illegible]. The AIB transport into uterine tissue precedes that into the blastocysts. The AIB transport [illegible] the blastocysts is maintained as long as they have [illegible] negative surface charge.

[illegible] in nutrient uptake activity that accompany changes in cellular proliferation [illegible] have been implicated in models of cell cycle control involving regulation of surface [illegible] functions (Holley 1972, Pardee 1974, Cecchini *et al.* 1976) However studies of [illegible] of transport rate of nutrients such as certain amino acids into intact cells [illegible] been difficult to interpret due to the complexity of the system. Thus, it has been dif

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Table I. Radioactivity of samples of buffer used for flushing and washing blastocyst in one representative experiment.

	Counts/10 min [illegible]
Flushing out [illegible] buffer including [illegible] [illegible] secretion	15
1st washing buffer	12
2nd washing buffer	10
[illegible] buffer	10

[illegible] microscopy preparation. Electron microscopy of the uterine epithelium was performed on [illegible] obtained from mice [illegible] had been influenced by estrogen for 8 h and by AIB for 4 h, and then [illegible] for blastocysts according to the standard procedure used in the experiments. Uterine horns from [illegible] were carefully fixed by an intraluminal injection of [illegible] solution of 2.5% glutaraldehyde in 0.1 M [illegible] buffer pH 7.2. The specimens were dehydrated in acetone and embedded in Westopal. One [illegible] from each animal [illegible] examined.

[illegible] for electron microscopy were obtained from 2 mice which had been given estrogen 8 h and AIB 4 h before the preparation. The blastocysts [illegible] flushed and incubated according to the procedure [illegible]. They were then fixed in [illegible] solution of 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, dehydrated in ethanol, and embedded in Epon. Two blastocysts from each animal were examined.

Results

The blastocysts demonstrated a conventional appearance without any sign of damage to the trophectoderm layer when inspected with the transmission electron microscope (Fig. 1). The ultrastructure of the uterine epithelium after the flushings was also normal (Fig. 2) indicating that no substantial amount of cytoplasmic content could have contaminated the flushing fluid. Both tissues were examined 8 h after the induction of implantation, with an in vivo incubation time of AIB for 4 h.

Further only the buffer used for flushing out the blastocysts contained measurable radioactivity while the final washing had background counting (Table I).

The in vivo transport of ^{14}C-AIB into the blastocysts is clearly seen 8 h after the induction of implantation while only a tiny transport occurs 12 h after the induction provided that the [illegible] incubation time was 4 h (Table II). No transport at all was measurable neither when the labeled amino acid was given concomitantly with the estrogen nor at any time [illegible] activation for implantation with an in vivo incubation time for only 1/3 h of ^{14}C AIB.

Table II. The uptake of ^{14}C-AIB in blastocysts in vivo

Number of blastocysts	Estrogen activation time, hours	^{14}C AIB incubation time, hours	Radioactivity of the blastocysts, counts/10 min
[illegible]	4	1/3	0
[illegible]	4	4	0
[illegible]	8	1/3	0
[illegible]	8	4	80
[illegible]	8	4	43
[illegible]	8	4	60
[illegible]	12	4	10
[illegible]	12	4	25
[illegible]	12	[illegible]	[illegible]

ficult to distinguish unambiguously between effects of intracellular metabolic changes, changes associated with the plasma membrane and effects of surface area on transport rates. Attempts have been made to separate uptake from subsequent metabolism by the use of non-metabolizable analogues, such as α-aminoisobuturic acid, which is transported by the system A as defined by Christensen (1975)

A favoured *in vivo* transport of 14C AIB was found to occur into mouse uterine tissue in estrogen-injected animals in delayed implantation provided that the estrogen injection preceded that of the 14C AIB by 4 to 8 h (Lindqvist *et al* 1977). Thus, during activation f implantation the first 8 h are a period when some amino acids are supplied from the blood of the mice into the uterine tissue at a high degree.

Since induction of implantation by estrogen administration is associated with an activation of the metabolism of the blastocyst (McLaren 1973) the uterine response could indicate an increased capacity to supply the blastocyst with amino acids. Therefore, the aim of the present studies was to examine whether also the blastocysts demonstrated a preferential transport of 14C AIB *in vivo* during activation for implantation.

Material and methods

14C-α-aminoisobuturic acid (1 14C AIB), Protosol, and Aquasol were purchased from NEN Chemicals GmbH, Dreieichenhain, W Germany and α-aminoisobuturic acid from Calbiochem, San Diego, USA. Depo-Provera was purchased from Upjohn s.a., Puurs, Belgium and estradiol-17-β from AB Leo, Helsingborg, Sweden. All chemicals used were analytical grade Radioactivity was measured in Nuclear Chicago Unilux II Liquid Scintillation Counter

Adult white female mice (N M R.I Anticimex, Stockholm, Sweden) were mated and the day the vaginal plug was found was recorded as day 1 The mice were spayed on day 3 and given a first dose of Depo-Provera (1 mg/0 0. ml) subcutaneously the same day to keep the blastocysts in delayed implantation (Humphrey 1967 Bergström 1977). A second dose was administered on day 10 or 11 and the animals were taken for experiments on day 11–13

In vivo incubation procedure Implantation was initiated by subcutaneous injection of 0.1 µg estradiol-17-β in 0.1 ml propylene glycol either concomitantly with or at different times prior to the administration of 14C-AIB The labeled amino acid (4.25 µCi 14C AIB in 0.1 ml of a 40 mM AIB-solution, made isotonic with NaCl) was given in the tail vein. The mice were anaesthetized by Nembutal and the uterus was removed within 5 min. The blastocysts were flushed out of the uterine horns with an ice-cold Krebs-Ringer choline-bicarbonate buffer (all sodium ions were stoichiometrically replaced by choline ions), freshly bubbled with a gas mixture of 93.5 % O_2 and 6.5 % CO_2 to give final pH of 7 4 The buffer also contained 100 mg bovine albumin/50 ml buffer to prevent stickiness of the blastocysts and to stabilize the blastocyst membranes (*cf* Kromphardt 1968). The buffer was ice-cold and Na-free in order to immobilize the AIB-carrier for transport.

The blastocysts were washed in the same ice-cold buffer as was used for the flushing out, transferred to fresh buffer counted and collected using a siliconized glass capillary and transferred to a weighed scintillation vial together with a small amount of buffer During this procedure the blastocysts displayed an unchanged appearance as observed in the preparation microscope. Buffer plus blastocysts were then weighed. Samples of the flushing out buffer the later washings and of pure buffer were also weighed in vials (Table I). All samples were dissolved in 1 ml of Protosol, 50 µl conc. acetic acid and 10 ml of Aquasol were added and the radioactivity was measured. Duration of blastocyst activation, 14C-AIB incubation, and the number of blastocysts in each experiment are shown in Table II

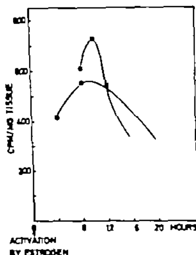
Uterine tissue samples for the retention experiments were treated as described before (Lindqvist *et al* 1977), that is the uterine horns were removed from the anesthetized animals, trimmed and washed in ice-cold buffer lightly blotted on filter paper weighed in scintillation vials and dissolved in Protosol over night. The solution was mixed with conc. acetic acid and Aquasol and the radioactivity measured Each retention value is the mean of the values derived from 3–6 animals (Fig. 3).



t of uterine epithelium from specimen obtained from mouse given estrogen 8 h and AIB 4 h preparation and then flushed for blastocysts according to the procedure used in the expts. The ...ure shown is representative of this type of epithelium demonstrating, among other things, an ...l epithelial lining with apical protrusions and conventional outline of the nuclei. The circular ...ly-stained areas are lipid granules. × 2 700.

...orbed onto the blastocyst surface nor had diffused into the blastocoelic fluid of ...l blastocysts. Considering that the second washing fluid never contained any measur... ...ity no leakage of labeled amino acid occurred from the blastocysts into the buffer ...e measured radioactivity of the isolated and washed blastocysts represented labeled ...cid taken up by the blastocysts.

...ransport ratio between blastocysts and uterine tissue were different for different ...n times indicating an autonomous transport of ^{14}C AIB for the blastocysts as com... ...th the uterus. The retention curves for labeled amino acid in uterine tissue displayed ...turnover when ^{14}C AIB was administered later during the activation for implanta-



...Retention of ^{14}C-AIB in mouse uterine tissue at dif... ...mes after induction of implantation. ●—●—● ^{14}C ...ministered 4 h after induction, ■—■—■ ^{14}C-AIB ...stered 8 h after induction. Max. stand. dev. ±30 cpm.

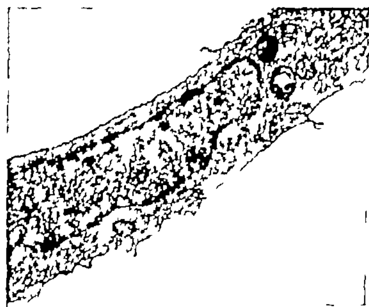


Fig. 1 Part of a blastocyst obtained from a mouse given estrogen 8 h and AIB 4 h before the preparation and incubated according to the procedure used in the expts. The ultrastructure shown is representative of this type of blastocysts demonstrating unchanged trophoblasts cells with normal-looking mitochondria. × 9 500.

The initial preferential uptake of ^{14}C-AIB in the uterus was higher at 8 h than at 4 h after the induction of implantation. The retention time was shorter when ^{14}C-AIB was given at 8 h than when given at 4 h (Fig. 3).

The transport ratio between blastocysts and uterine tissue

$$\left(\frac{\text{cpm/total number of blastocysts in each expt.}}{\text{cpm/mg uterine tissue}}\right)$$

was not maintained constant but grew from 0 at an activation time of 4 h to a maximum value of 0.011 at 8 h activation and then decreased to 0.003 after 12 h activation with an *in vivo* incubation time of 4 h in all expts. The discrepancy between uterine tissue and blastocysts at ratio 0 is further strengthened by the fact that the transport rate of ^{14}C-AIB into uterine tissue, is considerable already 1/3 h after the induction (Lindqvist *et al.* 1977) while still 4 h after the induction the transport into the blastocysts is not measurable (Table II).

Discussion

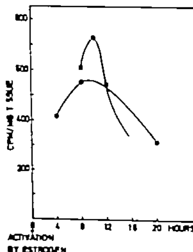
The blastocysts *in situ* displayed indeed under certain conditions an accumulation of ^{14}C-AIB administered *in vivo*. The peak value was found at 8 h after the injection of estrogen. At this time the blastocysts were not in direct contact with the uterine wall but lay in the uterine secretion (Nilsson 1974). Since the amount of radioactive amino acid in the uterine secretions surrounding the blastocysts was in excess of that taken up by the blastocysts and since the morphology of the tissues was normal, an adsorption of uterine epithelial fragments containing labeled amino acid onto the blastocysts hardly seems probable. This conclusion is strengthened by the expts. with the shorter *in vivo* incubation for only 1/3 h of ^{14}C-AIB. The radioactivity then was found in the flushing fluid while the washed blastocysts never contained any radioactivity. This indicates that AIB artifactually neither had



...rt of uterine epithelium from specimen obtained from mouse given estrogen 8 h and AIB 4 h ...e preparation and then flushed for blastocysts according to the procedure used in the expts. The ...tion show is representative of this type of epithelium demonstrating, among other things, an ...d epithelial lining with apical protrusions and conventional outline of the nuclei. The circular ...ly-stained areas are lipid granules. × 2 700.

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...transport ratio between blastocysts and uterine tissue were different for different ...on times indicating an autonomous transport of ^{14}C-AIB for the blastocysts as com... ...ith the uterus. The retention curves for labeled amino acid in uterine tissue displayed ... turnover when ^{14}C AIB was administered later during the activation for implanta-



...Retention of ^{14}C-AIB in mouse uterine tissue at dif... ...mes after induction of implantation. ●—●—● ^{14}C-... ...ministered 4 h after induction, ■—■—■ ^{14}C-AIB ...tered 8 h after induction. Max. stand. dev ± 30 cpm.

tion. This shorter retention time, when the radioactive amino acid was administered late during activation for implantation, explained the lower content of radioactivity in the blastocysts at 12 h after induction. Since the 8 h peak appeared at a time when the blastocysts are not in direct contact with the uterine wall it is reasonable to conclude that a vectorial transport of ^{14}C AIB took place from the uterine epithelium via the uterine secretion into the blastocysts, and that no simple diffusion mechanism exists between the uterine epithelium and the blastocysts. Further since the transport ratio between the uterine tissue and the blastocysts is not maintained constant it is concluded that the metabolic rates for amino acid transport are different for the uterus and the blastocysts. Reasonably the AIB transport into uterine tissue preceeds that into the blastocysts.

The space between the activated blastocysts and the uterine epithelium is filled with uterine secretion. This secretion contains the labeled AIB as well as other amino acids (Pratt 1977) Considering that *in vitro* expts. have shown the importance of some amino acids for the normal development of the blastocyst (Naeslund 1976), the system A and/or system L amino acids when reacting with their receptors at the blastocyst surface, can be working as trigger substances for blastocyst activation.

A parallelism exists between the *in vivo* transport of ^{14}C AIB into blastocysts and the surface charge of the blastocysts, since an uptake occurs as long as the blastocysts retain their negative surface charge (Nilsson *et al* 1975). Simultaneously with the loss of negative surface charge the *in vivo* transport of ^{14}C AIB into the blastocysts is also diminishing. This is interesting to note taking into account similar phenomena displayed by malignant cells, studied *in vitro* For instance, the negative charge on animal cell surfaces appears to be greater on several types of transformed cells as compared with their normal counterparts (Wallach and Eylar 1961 Forrester *et al* 1964 Kraemer 1966), and the ^{14}C AIB transport capacity of neoplastically transformed glioma cells was several times higher than that of glia cells (Ronquist *et al* 1976) Thus the activation of the mouse blastocyst has some similarities with the transformation of some strains of tissue culture cells. Often a direct relationship exists between the amount of sialic acid released by *e g* neuraminidase treatment and reduction of cellular electrophoretic mobility due to loss of negative charge at the cell surface (Weiss and Mayhew 1966) Since the removal of surface membrane sialic acid of HeLa cells resulted in a sharp decrease in ^{14}C AIB transport into these cells (Brown and Michael 1969) there might be a correlation between surface content of sialic acid and AIB transport. Hence the ^{14}C-AIB transport into blastocysts studied *in vivo* seems to have some characteristics as regards amino acid transport by the system A in common with other cell types studied *in vitro* However the blastocysts are able to control the uptake process displaying an AIB transport only as long as the negative charge is maintained (Nilsson *et al* 1975), while tumor cells seem not to be able to change their negative surface charge and consequently not the system A transport capacity

The presence of sodium ions is a necessary prerequisite for an active transport of ^{14}C AIB into the blastocyst. An increased content of Na in the endometrium of rats 18 h after induction of implantation was reported by Setty *et al* (1973) It was also claimed by Borland *et al* (1977) that the Na -movement into the blastocoele of mice might be energetically favoured at the approximate time for blastocyst activation. The sodium accumulation in-

[illegible] the blastocysts support the view that the blastocysts themselves contain system A carriers with a co-transport of Na^+ together with the ^{14}C AIB transport. Work on isolated mouse blastocysts for studies of the carrier function is under progress.

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Influence of indomethacin and of prostaglandin E_1 on total and regional blood flow in man

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Abstract

NOWAK, J and Å. WENNMALM. *Influence of indomethacin and of prostaglandin E on total and regional blood flow in man.* Acta physiol scand. 1978 *102* 484-491

The central and regional circulatory effects in man of the prostaglandin synthetase inhibitor indomethacin and of prostaglandin E_1 (PGE_1) were studied. Systemic blood pressure, cardiac output, and renal and splanchnic blood flow were measured at rest, following infusion of indomethacin (50 mg i.v.), and during infusion of PGE (4–8 μg min l.) after the administration of indomethacin. An increase in the total systemic resistance (+20 %), as well as in the renal (+30 %) and splanchnic (+16 %) vascular resistance developed rapidly following the administration of indomethacin. Infusion of PGE completely restored the resistance in the renal and splanchnic regions, and in addition markedly increased the blood flow in non-visceral tissues. We suggest that the circulatory effects by indomethacin are elicited via the drug's inhibitory effect on prostaglandin synthetase in the vessel walls, and that vasodilating products of PG synthetase affect the regional blood flow distribution in man.

It has been known for at least forty years that some of the prostaglandins decrease the systemic blood pressure (Euler 1936) This pharmacological action by PGs, well documented in isolated preparations as well as in intact animals of different species, has not yet been convincingly shown to have a physiological equivalent Apparently the lack of receptor inhibitors for the action of PGs on vascular smooth muscle has hampered the demonstration of possible effects of endogenously formed PG in the circulatory system. With the discovery by Vane (1971) that certain anti-inflammatory drugs inhibit the bioformation of PGs, a tool was provided for demonstrating the contribution of endogenous PGs in physiological mechanisms. Thus, following infusion of the PG synthetase inhibitor indomethacin, an increase in the systemic blood pressure was observed in rabbits (Änggård and Larsson 1973) as well as in dogs (Lonigro *et al.* 1973), possibly due to an inhibited formation of vasodilating PGs in the vascular bed of the animal. The observation was later confirmed in man (Wennmalm 1974 Patak *et al* 1975), inasmuch as infusion of indomethacin i.v. was followed by a decrease in cardiac output and a parallel increase in the systemic blood pressure. These data thus indicated that indomethacin increases the systemic vascular resistance in man.

In the present study the possible influence of vasodilating PGs in the regional blood flow distribution in man has been studied further. First, the regional blood flow changes following administration of indomethacin were measured, and second, the ability of PGE infused i. to restore these blood flow changes caused by indomethacin, was investigated.

Methods

The study was performed with the permission of the Ethical Committee at the Karolinska Institute. 19 healthy men, aged 23–41 years, participated. They were all informed of the nature, purpose and possible risks of the investigation before giving their voluntary consent to take part in the study. All investigations were performed in the morning after an over-night fast.

In 10 of the volunteers, the following experimental procedure was performed. A Swan–Ganz catheter (no. 7) was inserted percutaneously into a medial cubital vein or into the right femoral vein and passed under fluoroscopic control to the pulmonary artery. Two Cournand catheters (no. 7 or 8) were inserted similarly and passed to a right-sided hepatic vein and to the right or left renal vein, respectively. Two short teflon catheters were inserted into a superficial forearm vein and a brachial artery respectively. Pressure recordings were performed via a pressure transducer (Elema EMT 35) on a photochymograph (Abem Ultralette). Expired air was collected in Douglas bags and analyzed by the Scholander microtechnique. Blood samples were drawn from the brachial and pulmonary arteries for determination of oxygen content. The cardiac output (C.O.) was measured using the direct Fick method.

A mixture of indocyanine green dye (Cardio-Green, HW & D) and paraaminohippurate (Sodium aminohippurate, MSD) was infused into the short venous forearm catheter. Simultaneous arterial and hepatic and renal venous blood samples were drawn at regular intervals for determination of dye and PAH concentrations. Splanchnic and renal blood flows were estimated using the constant infusion technique (Bradley [illegible]).

Indomethacin (MSD, purity > 99 %) was dissolved in ethanol at a concentration of 5 mg/ml and passed through a sterile filter. Immediately prior to the infusion, 10 ml of this solution was added to 25 ml of sterile 0.1 M phosphate buffer, pH 7.5. The mixture was infused at a rate of 1–2 ml/min via the Swan–Ganz catheter. Sterile prostaglandin E_1 was obtained from Astra Läkemedel AB. It was dissolved in ethanol at a concentration of 100 μg/ml. Immediately prior to the infusion it was diluted in saline to a final concentration of 4 μg/ml. This solution was infused via the Swan–Ganz catheter at a rate of 1 or 2 ml/min.

During the experiments, the subjects rested comfortably in the supine position. After the first 35 min of the PAH infusion, during which the plasma levels of these indicators stabilized, blood pressure and total and regional flow measurements were performed twice with a 25 min interval. Subsequently indomethacin was infused as described above. After the end of the indomethacin infusion, blood pressure and flow measurements were repeated twice with a 25 min interval. Thereafter PGE_1 infusion was started at a rate of 4 μg/min. In 4 subjects, who did not display any decrease in blood pressure after 5 min of PGE_1 infusion at 4 μg/min, the infusion rate was elevated to 8 μg/min. The total time of infusion of PGE_1 was 25–40 min. Total and regional flow measurements were started after 15 min of PGE_1 infusion. The measurements were repeated as close as possible before the discontinuation of the infusion of PGE_1.

The remaining 9 subjects served as controls and were subjected to a simplified experimental procedure, wherein only the cardiac output and the systemic blood pressure were estimated, at rest and following infusion of indomethacin solvent.

Calculations. Non-visceral blood flow was estimated by subtracting the actual figures on renal and splanchnic flow from the cardiac output. Data in the text and figures are expressed as mean ± S.E. Figures within brackets indicate the number of expts. Students' t-test for paired differences has been used when applicable.

Results

1. Central and regional blood flow before drug administration

At supine rest before the infusion of drugs, all circulatory and respiratory parameters recorded were well within the normal limits. The cardiac output (6.5 l/min) was distributed to the tissues as follows. 26 ± 2 % (9) perfused the kidneys, 19 ± 1 % (10) passed through the

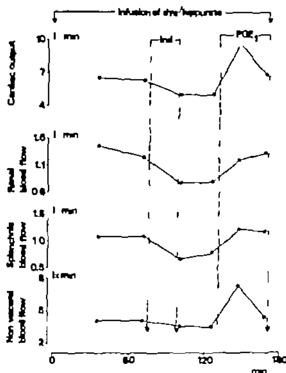


Fig. 1 Typical experiment demonstrating the effect of indomethacin (IND 50 mg infused i.v. during 20 min) and of prostaglandin E_1 (PGE 160 μg infused i.v. during 40 min) on cardiac output, and on renal, splanchnic, and non-visceral blood flow in a healthy male volunteer aged 3...

splanchnic region, and the remaining 55 ± 3 % (9) was thus directed through the non-visceral tissues.

b Effect of indomethacin on the central and regional circulation (Fig. 1 2)

Within 2–3 min of starting the infusion of indomethacin, increases in the systolic and diastolic blood pressures were observed. The elevation of the systemic blood pressure was fully developed within 10–15 min of infusion and persisted until PGE was infused (see below) Since cardiac output was insignificantly affected the increased systemic blood pressure must have been due to an increased overall vascular resistance in the systemic circulation. In fact the systemic vascular resistance was increased significantly by 20 ± 8% (10). However the regional resistances were not equally affected by indomethacin Thus significant increases were obtained in the renal vascular resistance (by 30 ± 9 %, n = 9) and in the splanchnic resistance (by 16 ± 6 %, n = 10), while the vascular resistance in the non-visceral organs was insignificantly affected Oxygen uptake and R value did not differ from resting values, indicating that the drug did not induce any metabolic effects.

In the 9 subjects infused with indomethacin solvent only no changes were observed in systemic vascular resistance (93 ± 8 kPa 1 s before and 97 ± 7 kPa 1 s after the infusion).

In no case did infusion of indomethacin or solvent elicit any kind of discomfort in the subjects. No side effects of the drug were observed at reexamination 1–2 weeks after the investigation.

c Effect of PGE on the central and regional circulation (Fig. 1 3)

During continuous infusion of PGE at a rate of 4–8 μg/kg b.w./min after indomethacin ... hyperkinetic circulation appeared, with increased cardiac output and decreased A-V O...

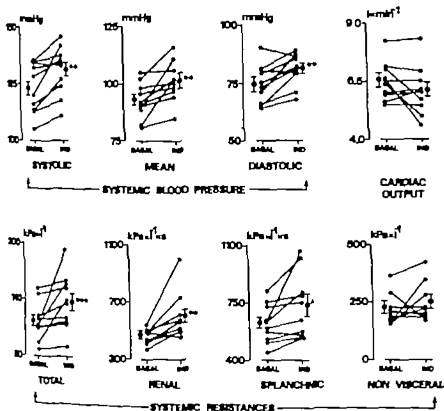


Fig. 2. Systemic blood pressure, cardiac output, and regional vascular resistances at rest (BASAL), and following administration of 50 mg of indomethacin (IND.). The smaller symbols represent the individual observations and the larger symbols indicate mean ± S.E. in the respective groups. *, ** or *** indicates that the mean differs significantly (P < 0.05, P < 0.01 or P < 0.001, respectively) from the corresponding value before indomethacin.

difference (from 45 ± 2 ml/l before to 33 ± 2 ml/l during the infusion, n = 10). The systemic blood pressure returned to the level observed before the infusion of indomethacin, and consequently the increased cardiac output was paralleled by a decreased overall systemic vascular resistance. The estimated systemic resistance was decreased by 23 ± 6% (10) com-

Table 1. Regional blood flow before indomethacin and during an i.v. infusion of PGE_1 after administration of indomethacin (50 mg). The values are presented as mean ± S.E. Figures within brackets indicate number of expts.

	Renal blood flow ml min⁻¹	Splanchnic blood flow ml min⁻¹	Non-visceral blood flow ml min⁻¹
Basal	1 610 ± 70 (9)	1 220 ± 70 (10)	3 480 ± 250 (9)
During inf. of PGE_1 ([illegible])	1 640 ± 100 (10)	1 210 ± 70 (10)	5 830 ± 660 (10)

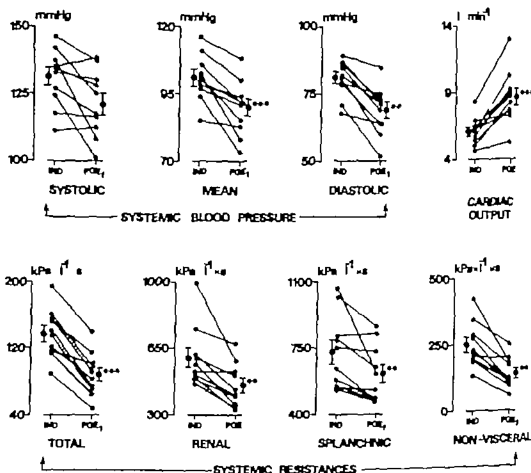


Fig. 3. Systemic blood pressure, cardiac output, and regional vascular resistances 25 min after administration of indomethacin (IND 50 mg), and during infusion of prostaglandin E_1 (PGE_1 4 µg/min). The smaller symbols represent the individual observations and the larger symbols indicate mean ± S.E. in the respective groups. ** or *** indicates that the mean differs significantly ($P < 0.01$ or $P < 0.001$, respectively) from the corresponding figure before PGE_1.

pared with before indomethacin. The increased cardiac output was not distributed uniformly to the different organs. In the kidneys and the splanchnic region, the flow was increased no more than to the resting level observed before indomethacin (Table I). Since the cardiac output was augmented beyond this level, the non-visceral tissues must have received a considerably higher flow during PGE_1 infusion than before infusion of indomethacin (Table I). The estimated local vascular resistances verify the uneven increases in flow during PGE_1 infusion: the resistance in the non-visceral tissues was decreased by 29 ± 12% (9), while the renal and splanchnic vascular resistances were unchanged compared to the values before indomethacin. No changes in the oxygen uptake or the R value were observed, indicating that PGE_1 in the present dose lacks general metabolic effects.

In one subject, infusion of PGE_1 (8 µg/min × kg b.w.) elicited abdominal pains, which disappeared following immediate discontinuation of the infusion. The remaining subjects

Discussion

[illegible]ion of indomethacin in the current experiments induced distinct circulatory effects. [illegible] unchanged circulation following the infusion of drug solvent only revealed that indo[illegible]thacin was the active principle and that the solvent ethanol was in itself without effect. [illegible]istered indomethacin elicited an increase in blood pressure parallel with an unchanged or [illegible]ghtly decreased cardiac output. These changes in the direction of a more hypokinetic [illegible]ulation indicate that the primary effect of the drug was to increase the peripheral re[illegible]tance. The increase in resistance was more prominent in the kidneys than in the remaining [illegible]t of the systemic circulation. The varying effect of indomethacin in the different re[illegible]tance regions suggests that the drug effect was selective and not due to a general (central) [illegible]oconstrictive action in the systemic resistance bed. It therefore appears that indomethacin [illegible]ed peripherally possibly by interfering with local vasoregulatory mechanisms. Indo[illegible]ethacin is a well-known inhibitor of the biosynthesis of prostaglandins (Vane 1971). Since [illegible]ny PGs, e.g. those of the A, E, G and I series) are vasodilators, the currently observed [illegible]temic vasoconstriction caused by a PG synthetase inhibitor may have been due to the [illegible]thdrawal of endogenous PGs from certain parts of the systemic resistance vascular bed. [illegible]omethacin has recently been shown to inhibit PG synthesis in man considerably at a [illegible]sma drug concentration of 0.1 μg/ml (Rane *et al.* 1977). Assuming an indomethacin [illegible]ace of 50 l in the subjects in the present study a drug concentration of 0.1 μg/ml would [illegible] reached after 2–3 min of infusion. Thus, the currently observed rapid onset (within 2–3 [illegible]n) of the blood pressure increasing effect of indomethacin seems plausible with respect [illegible] inhibition of PG synthesis.

It has earlier been shown in dogs (Lonigro *et al.* 1973) as well as in rabbits (Colina-Chourio *et al.* 1975), that the renal vascular tone is counteracted by intra-renally formed PGs and [illegible] inhibition of their synthesis is followed by an increase in the systemic blood pressure. [illegible]e current results, which clearly demonstrate an increased vascular resistance following [illegible]fusion of indomethacin, extend these observations to the supine, un-anesthetized man. [illegible]owever the currently observed effect of indomethacin on the human renal blood flow was [illegible]siderably less than that obtained in the above-mentioned dog study (Lonigro *et al.* 1973). The observed effect of indomethacin in the splanchnic region was smaller than the cor[illegible]esponding drug action in the kidneys. Nevertheless it deserves attention since an effect of [illegible]omethacin, which may reflect synthesis of endogenous vasodilating PGs in the splanchnic [illegible]scular bed, has not been observed before. The resistance in the non-visceral tissues, which [illegible]ainly represent skin and skeletal muscle, was not affected in the present study. This is [illegible]sistent with earlier observations, demonstrating an unchanged resting skeletal muscle [illegible]lood flow following indomethacin in dogs (Herbaczynska-Cedro *et al.* 1973) and in humans [illegible]lbom and Wennmalm 1976).

[illegible]fusion of PGE, completely reversed the resistance effect of indomethacin in the kidneys [illegible]d the splanchnic region, and in addition markedly decreased the non-visceral resistance. [illegible]e resistance vessels in the various regions were thus all relaxed when exposed to PGE, [illegible]t to different degree. This indicates that the resistance changes caused by the preceding [illegible]fusion of indomethacin were not only—if at all—induced by the withdrawal of endo-

genously produced PGs of the E series from the vascular bed. Other vasodilating PGs, like those at the A, G and I series, the synthesis of which is also inhibited by indomethacin, may have contributed to the lower "pre-indomethacin" resistance as well. Of special interest in this connection is the newly discovered PGI (Gryglewski *et al.* 1976, Bunting *et al.* 1976) which is a potent vasodilator and seems to be selectively formed in vessel walls. Armstrong *et al* (1977), infusing PGI i.v in the anesthetized open-chest dog demonstrated a decrease in systemic blood pressure and total peripheral resistance. Analysis of the regional effects of PGI_2 have, however not been reported hitherto

Interestingly the vascular bed that displayed the highest sensitivity to PGE_1, *i.e* the non-visceral tissues, was insignificantly affected by indomethacin. Although the lack of effect of indomethacin on the non-visceral resistance implies that no production of vasodilating PGs was going on in these vessels during the present experimental conditions, the high vascular sensitivity to PGE in this region may still be of significance. Much attention has recently focused on the possibility that endogenous vasodilating PGs may take part in the development of reactive hyperemia in coronary vessels (Kent *et al* 1976, Afonso *et al* 1974) as well as in skeletal muscle (Kilbom and Wennmalm 1974 Messina *et al.* 1974). The high vascular sensitivity to PGE in the non-visceral tissues observed in the current experiments constitutes a beneficial basis for the significant contribution of endogenous PGs in developing muscle reactive hyperemia.

To summarize, the present investigation demonstrates regional differences in the increase in peripheral resistance in man following administration of the prostaglandin synthetase inhibitor indomethacin. Furthermore, it demonstrates that the degree of vascular relaxation to PGE varies between regions. On the basis of these observations it is suggested that tissue or organ differences in vascular resistance in man may depend to some extent on locally formed vasodilating PGs.

This study was supported by the Swedish Medical Research Council, project 04X-4341 and by Magnus Bergvalls Stiftelse. Indomethacin was kindly put t our disposal by the Merck Sharp & Dohme Company through its swedish agent Erik Lindblom & Co KB. PGE_1 was kind gift from Astra Läkemedel AB.

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Programmed pattern of muscular activity in monkeys landing from a leap

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Muscular activity begins before the foot touches the ground in both stepping (Engberg an Lundberg 1969) and sudden falls (Melvill Jones and Watt 1971 Greenwood and Hopkin 1976, Prochazka *et al* 1977) The activity absorbing the impact of landing, thus, is no elicited by impulses from receptors in the leg. After landing the activity may either be cor trolled by a program, by local or central reflexes, or it may be of mixed origin. The aim of th study was to determine if reflexes take part in the control of activity occurring immediatel after landing.

4 monkeys were trained to jump from one platform to another placed from 50 to 165 c beneath it. The height of the starting platform in successive leaps was varied in a predete mined random sequence. Thus, a stereotyped movement pattern could not replace regulate onset of anticipatory contraction Each jump was rewarded with a pellet of food and a r cording session consisted of about 40 jumps. The electrical activity of the extensor (m. tricep brachii) and flexor (m. biceps brachii) was recorded with wire electrodes.

A third recording channel displayed the output from a piezoelectric force transducer in th landing platform. Strain on the wires from the animal to the recording equipment was pr vented with contacts rotating in grooves filled with mercury and a spring loaded aluminur rod carried the wires. The difference between the acceleration of the free fall and the acceler tion retarded by the wire-carrying device was less than 5%.

Electrical activity in the triceps began 38 ms before landing and the initial extensor burs lasted until about 10 ms after contact with the landing platform. Subsequently 3 or 4 burst each lasting 12 ms were separated by silent periods of 10 ms. Electrical activity in the bicep began 30 ms before the hands touched the platform but the burst ended 10 ms before contact Subsequently extensor and flexor activity occurred in a reciprocal pattern (Fig. 1).

The time of onset counted from the time of landing was independent of the height of th jump. The onset thus occurred at greater heights the higher the jump. The probability tha the monkey turned the extensor on at a constant time after departure from the starting plat form was calculated from linear regression analysis and found to be less than 0 001

The timing and the pattern of the muscular activity were independent of the height of th jump This is compatible with the release of a programmed pattern of responses (Chan and Kearney 1977).

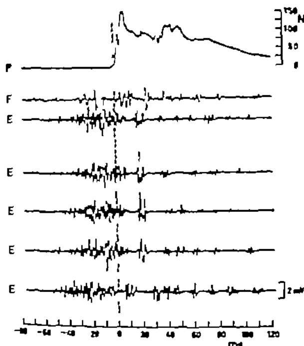


Fig. 1 Landing force (P) and EMG in arm flexor (F) and extensor muscles (E), during landing from leap. Upper EMG recordings are from leaps from 120 cm. The lowest recording is from leap to a paper cylinder of the same height. The monkey went through the paper platform and made solid landing after falling another 8 cm. Notice that the first burst of muscular activity moved forward in time in relation to the landing, indicating that the monkey was deceived. The first period of silence in the extensor now started before landing and thus was not of reflex origin. The second burst of activity also moved forward in time indicating that it was controlled or influenced by the motor program.

To determine if the first silent period in the extensor discharge was reflex inhibition we placed an 8 cm high, solid black cylinder on top of the landing platform and let the monkey jump 10 times. In the subsequent trial the solid cylinder was replaced with another of the same appearance, but made from tissue paper. The lowest recording of Fig. 1 shows that the extensor activity now began about 50 ms before the impact on the landing platform. The time, measured from high-speed cinematography for the 8 cm fall from the top of the paper cylinder to the landing platform was 16 ms. This means that the activity started about 35 ms after the hands arrived at the soft paper cylinder. The deception moved not only the time of onset of the extensor forward in time, but the onset of silence moved with it and now occurred before the impact. Hence, this part of the pattern was not a reflex.

The functional significance of the EMG pattern after landing is uncertain. Nichols and Houk (1976) found that the reflex mediated peak of EMG activity occurring just after the onset of muscle stretch was associated with a huge increase in stiffness of the lengthening muscle. The basis of comparison of stiffness was the same muscle under steady motorneuronal drive. It is possible that the programmed pattern observed by us after landing also is associated with high muscular stiffness.

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From the Department of Pediatrics, University Hospital, Uppsala and Departments of Pharmacology and Chemistry Karolinska Institute, Stockholm, Sweden

Contractile action of a stable prostaglandin endoperoxide analogue on the human umbilical artery

By

TORSTEN TUVEMO, KJELL STRANDBERG and MATS HAMBERG

Received 4 January 1978

…staglandin (PG) endoperoxides PGG and PGH are potent contractors of the … human umbilical artery (Tuvemo *et al.* 1976). During PG biosynthesis they are converted to PGs, *e.g.* PGE$_2$, PGF$_{2\alpha}$ and PGI (prostacyclin) and to thromboxane A$_2$ (Hamberg *et al.* 1975). As TxA$_2$ is an even more potent contractor of the human …al artery than the PG endoperoxides it is possible that the effects produced by the …mpounds in fact reflect the formation of TxA (Svensson *et al.* 1977). The recent …s of 15(S)-hydroxy-9α,11 -(epoxymethano)prosta-5,13-dienoic acid (EPA), a stable …doperoxide analogue, offers a tool for evaluation of the biological effects of the PG …roxides *per se*.

…is study we have compared the contractile effects of EPA and PGE$_2$ on spiral strips …an umbilical arteries by studying the cumulative dose-effect relations for the respec… …mpounds. The strips were prepared immediately after cord clamping, and mounted …n baths containing Krebs-bicarbonate-glucose solution at 37°C, pH about 7.4, …d pCO approximately 100 mmHg and 40 mmHg respectively (Strandberg and …a 1975). The preparations were allowed to stabilize at the level attained spontaneously …testing was begun. Drugs were added to the bath at 3 min intervals until maximal …e as produced. Both drugs were dissolved in the salt solution used prior to testing. …administration to the bath EPA produced a prompt, sustained contraction of the …d human umbilical artery In contrast the response to PGE$_2$ was slower in onset. As …y has been shown (Tuvemo *et al.* 1976), the response to the endoperoxides (PGG …GH$_2$) is also rapid in onset, but less sustained.

… cumulative dose-response curves for EPA and PGE$_2$ are shown in Fig. 1 ED$_{50}$ for …was approximately 3 10–[illegible] mol/l as compared to about 1.5 10–[illegible] mol/l for PGE$_2$, *i.e.* …-fold difference in activity

…s EPA is an extremely potent contractor of the isolated human umbilical artery In …spect it is more potent than all other stable compounds tested, including 5-HT In …on the present results [illegible] that previously recorded responses using the PG endo-

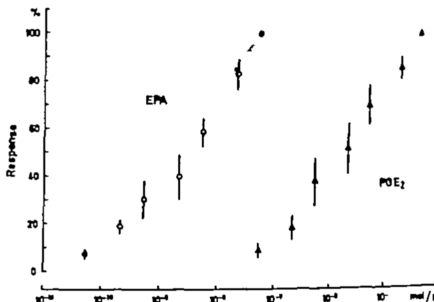


Fig. 1 Cumulative dose response curves for EPA (N = 6) and PGE₂ (= 6). Means ± S.D are given.

peroxides PGG and PGH at least partly reflect the action of the compounds proper not entirely the conversion product TxA_2.

In analogy with these findings EPA has been reported to be markedly more active PGG and PGH in a number of other smooth muscle preparations and in raising pulmonary arterial pressure in the cat and the airway insufflation pressure in the guinea (Chapnick *et al* 1976, Hedqvist *et al* 1977)

This study was supported financially by a grant from Stiftelsen Samariten, Stockholm.

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Abstracts from Meeting of the Scandinavian Physiological Society in Lund 25–26 November 1977

COMMUNICATIONS

C 1

-dependent Myogenic Microvascular Control

By P-O GRANDE and S MELLANDER. *Department of Physiology University of Lund S eden*

Bayliss' hypothesis of enhanced contractile activity in vascular smooth muscle in response to increased blood pressure has received strong support from hemodynamic ...organ and vital microscopy studies. The described myogenic responses, however ...almost exclusively been studied in terms of the active constrictor/dilator responses ...ch develop upon a given steady state increase/decrease in vascular transmural pres... ...and, hence, can be considered to reflect "static" myogenic reactivity.

...n additional dynamic or rate-sensitive, component in vascular myogenic control ...recently demonstrated *in vitro* by observations of electrical and mechanical activity ...he rat portal vein in response to a given static stretch or shortening applied at various ...s (Johansson and Mellander 1975). Dynamic stimuli were much more effective in ex...ng or inhibiting the vascular smooth muscle than static stimuli. This regulatory prin...le seems to apply to the microvessels *in vivo* as well as revealed by studies on the ...cular bed of skeletal muscle exposed to a given change of transmural pressure applied ...two different rates (Grände *et al.* 1977).

These stimulus-effector characteristics were analysed in greater detail in the present ...dy by observations of total and microvascular resistance responses to graded changes ...vascular transmural pressure (P_T) applied at different rates (dP_T/dt) over the entire range ...m +7.5 to −7.5 mm Hg/s. The rate-sensitive microvascular responses, developing ...ring the phase of changing P_T, were distinctly graded in relation to the magnitude of ...e dP_T/dt stimulus, both with regard to the amplitude of the resistance response and the ...te of resistance change per unit time (dR_{micro}/dt). The static microvascular responses ...corded in the steady state phase of constant increased P_T were small compared to the ...namic ones and graded in relation to the amplitude of the P_T increase. Rate-sensitivity ...microvascular control was bi-directional causing constriction in response to positive, ...d dilation in response to negative values of dP_T/dt. The data further indicated that the ...ferent myogenic receptor units operate with different thresholds. The described rate-...nsitivity in the myogenic control system seems to increase its rapidity, stability and ...sitivity and thereby can contribute efficiently to well-adapted and refined microvascular ...ustments.

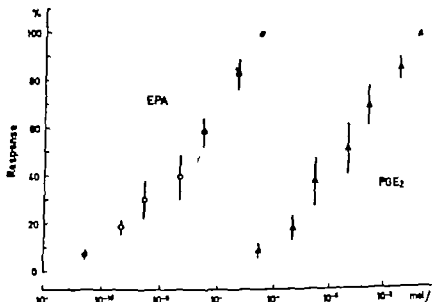


Fig. 1 Cumulative dose response curves for EPA (N = 6) and PGE, (n = 6). Means ± S.D are sh

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COMMUNICATIONS

C 1

...pendent Myogenic Microvascular Control

...y P.-O. GRÄNDE and S. MELLANDER. *Department of Physiology, University of ...und, S...eden*

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C 2

Potassium Permeability of the Mesothelium of the Frog Mesentery

By J FRØKJÆR JENSEN and O CHRISTENSEN *Institute of Medical Physiology Dept A The Panum Institute University of Copenhagen Denmark*

The mesothelium covering the mesentery has been claimed to possess diffusional proper ties equivalent to capillary endothelium and the *in vitro* preparation of the isolated mesen tery has been used as a model of the endothelium (Rasio 1974)

We have determined the K^+ permeability of the mesothelium of the frog mesentery *in vivo* The K^+-concentration of the interstitial tissue is increased by a continuous perfusion through a single capillary with an isotonic Ringer s solution containing an elevated K concentration. When steady-state is achieved the perfusion is stopped and the wash-out of K across the mesothelium followed by use of K sensitive microelectrodes inserted into the interstitium at various distances from the capillary (Fig. 1)

After an initial lag time the wash-out curves show a monoexponential decline of K concentration. The K -permeability of the mesothelium is then determined from the re lation. $P_K = d/\tau$ where τ is the decay time and d is the thickness of the interstitium. We found a $P_K = 2.4 \pm 0.8$ 10^{-6} cm sec (S D n = 10) a value about 25 times lower than the K -permeability of the mesenteric capillaries (Crone Frokjær Jensen Friedman and Christensen 1978) and about 30 times lower than the values previously reported from *in vitro* determinations This large discrepancy could be due to damage of the mesentery during *in vitro* mounting (Tesi Detraz and Forsmann 1971)

We conclude that in the frog the mesothelium of the mesentery differs significantly fr

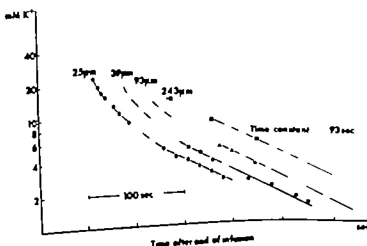


Fig. 1 K wash-out from the mesentery The decay of the interstitial K -concentration is shown at various distances from a perfused capillary through which the intersti tium had been loaded with potassium After an initial lag time the decay in K concentration is monoexpo nential at all distances. The time constant for wash-out was 93 sec.

[illegible]chemical properties from the endothelium of the capillary and thus represents a poor [illegible]odel of the capillary wall. Furthermore, the findings stress the importance of the meso-[illegible]helium as a diffusion barrier—a fact which may be critical in experiments on osmotic [illegible] movement across the capillary wall induced by hypertonic solutions on the surface [illegible] the mesentery (Landis and Sage 1971, Curry, Mason and Michel 1976)

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C 3

[illegible] Capillary Volume in Rat Hindquarter Measured with Gravimetric Method

By A. KAMIYA, B. RIPPE and B. FOLKOW. *Department of Physiology, University of Göteborg, Sweden*

[illegible] attempts have been made, particularly with morphological technique, to estimate [illegible] capillary volume in tissues. In the present study a physiological approach was uti-[illegible] in an "isogravimetric" rat hindquarter preparation, so arranged that weight changes [illegible] vascular distension or recoil were automatically balanced off. The volume of the [illegible]lary exchange vessels could then be deduced from the weight change, caused by [illegible] transient fluid absorption into the capillaries when inflow was suddenly stopped.

[illegible] matched hindquarter preparations were perfused in parallel with a plasma substi-[illegible] at constant total flow and maximal dilatation while total weight was continuously [illegible]rded. Starting from isogravimetric conditions one of the arterial inflows was suddenly [illegible]ped so that the doubled inflow to the other preparation here caused prompt vascular [illegible]ension followed by steady transcapillary filtration. In the occluded preparation a pas-[illegible] vascular recoil occurred, followed by a transient absorption of tissue fluid into the [illegible] post capillary contents. Arterial and venous outflow pressures were initially so set [illegible] the transient vascular distension and recoil events largely cancelled out between the [illegible] preparations. Upon the unilateral flow interruption the steady filtration in the un-[illegible]ded vascular bed showed up first after a distinct delay, which was due to the transient [illegible] absorption in the occluded vascular bed. From this time delay Δt, and the final out [illegible] filtration rate $\dot{Q}$, the absorbed fluid amount Q was obtained by $\dot{Q}\Delta t$. Capillary [illegible]lume $\dot{V}c$ could then be calculated by

$$\dot{V}c = Q/\ln[\Pi c/(\Pi c - Pc - Pv)]$$

[illegible]ere Πc, Pc, Pv represent plasma colloid osmotic pressure, capillary pressure and ve[illegible] outflow pressure during control isogravimetric conditions.

Capillary volume thus obtained amounted to 0.55–0.60 per cent of hindquarter weight

where total vascular volume at maximal dilatation is around 3–4 per cent. The approximate capillary number in hindquarter skeletal muscle was also deduced from these data and from average capillary diameter A figure of 450–500/mm² cross-sectional area was obtained in good agreement with morphological counts in skeletal muscle.

The present approach may be useful particularly in composed tissues of complex design where other methods meet with difficulties and where one wants to know the volume of the "*functional*" capillary vessels i e of the microvascular sections allowing a substantial exchange across the walls which then includes venular parts

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C 4

Capillary Permeability: Is the Pappenheimer–Karnovsky Pore Model Correct?

By WILLIAM P PAASKE *Institute of Medical Physiology B University of Copenhagen Denmark*

Capillary permeability of the hydrophilic indicators ^{51}Cr-ethylene-diamine-tetraacetat ^{51}Cr EDTA ^{57}Co-cyanocobalamin ^{57}Co-B12, and Inulin-(^{14}C)carboxylic acid was determined in rabbit adipose and cutaneous tissues and cat gastrocnemius muscle as indicated in Table I The single injection residue detection (alternatively venous sampling) technique and computerized semi on-line black-box analysis were used

The permeability coefficients P_d were calculated from the capillary diffusion coe

TABLE I. Capillary permeability in adipose tissue cutaneous tissue and skeletal muscle.

		Adipose tissue	Cutaneous tissue	Skeletal muscle
CDC(^{51}Cr EDTA)	ml/100 g min	2 0 (n=6)	3 7 (n=6)	3 8–9 1 (n=16)
CDC(^{57}Co-B12)	ml/100 g min	1 1 (n=6)	2.3 (n=6)	1 6–4 4 (n=16)
CDC(^{14}C-inulin)	ml/100 g min			0.84 (n=7)
P_d(^{51}Cr EDTA)	cm/s	0 95 10^{-5}	0.88 10^{-5}	0.74 10^{-5}
P_d(^{57}Co-B12)	cm/s	0.52 10^{-5}	0.56 10^{-5}	0.45 10^{-5}
P_d(^{14}C-inulin)	cm/s			0.20 10^{-5}
CDC(^{51}Cr EDTA)/ CDC(^{57}Co-B12)		1.82	1.61	1 79
CDC(^{51}Cr EDTA)/ CDC(^{14}C-inulin)				3.21
CDC(^{57}Co-B12)/ CDC(^{14}C-inulin)				2 04
D(^{51}Cr EDTA)/ D(^{57}Co-B12)	1 79			
D(^{51}Cr EDTA)/ D(^{14}C-inulin)	3 17			
D(^{57}Co-B12)/ D(^{14}C-inulin)	1 76			

Abbreviations. CDC capillary diffusion coefficient. P_d permeability coefficient. D free diffusion coefficient in water at 37°C.

ficients, CDC, and estimates of capillary surface area (adipose tissue: 35 cm^2/g, cutaneous tissue and skeletal muscle: 70 cm^2/g)

In skeletal muscle, CDC increased with increasing plasma flow Considering P constant, a 3-3.5 fold increase ("recruitment") of effective exchange area was found with a plasma flow increase from 4.0 to 52.4 ml/100 g min.

The indicators permeated the endothelial barrier of the continuous capillaries at rates proportional to their free diffusion coefficients in water at 37°C. This is taken as evidence for the conclusion that restricted diffusion did not occur

The findings are in agreement with those obtained in skeletal muscle by the indicator diffusion method (Crone 1963). They imply a much larger pore size than the 30 Å equivalent pore radius estimate obtained with the osmotic transient method (Pappenheimer et al. 1951) which is complicated by osmotic reflection coefficients corrections. The basic assumption of Pappenheimer of a well-mixed interstitial compartment is not supported by the present series, as the washout of indicator from the interstitium does not follow a monoexponential function.

The Pappenheimer–Karnovsky (1967) concept of aqueous interendothelial parallel plate channels (clefts) with a 37 Å slit width as the morphological pathway for transcapillary exchange of hydrophilic solutes is not supported by the present findings. The transendothelial channel system formed dynamically by random fusion of intraendothelial vesicles (Simionescu et al. 1975) seems a possible structural equivalent.

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C 5

The Temperature Dependence of the Latency Relaxation in Isolated Single Frog Skeletal Muscle Fibres

By P Haugen *Department of Biophysics University of Copenhagen, Denmark*

The latency relaxation (LR) was studied at sarcomere lengths (S) in the range 2.2–3 7 μm and at temperatures from 0 to 23°C in order to shed some light on what processes might be involved in generating this phenomenon.

Materials and methods were basically the same as those previously described (Haugen and Sten-Knudsen, 1976). The LR was quantified in terms of the values defined in the insert of Fig. 1.

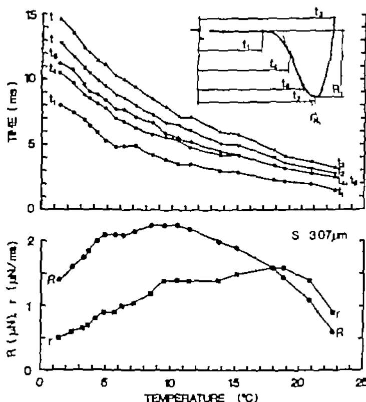


Fig. 1 Explained in the text.

Two types of experiments were performed. *1*) the values were measured as fu tions of *S* while the fibre was kept at constant temperature *R* passed through a maxim which occurred at higher *S* the lower the temperature. r_R showed a similar depende on *S* but its maximum was less sharp than that of *R* At temperatures below about 12° portion (from t_4 to t_a) of the falling phase was linear At higher temperatures t_4 an merged into an inflexion point. This can be interpreted as if LR originates from a len ening process which eventually is masked by the contraction. At all temperatures intervals from t_1 to t_a t_3, and t were proportional to the distance from the Z-line to overlap zone thereby indicating that the lengthening originates from the thin filame t_4 when separate from t_a was independent of *S* up to about 3 μm while it increased w *S* above that. *2*) The values were measured as functions of temperature at const *S* The results of such an experiment are shown in Fig 1 The processes had a Q_{10} of ab 2 as obtained from the time parameters *R* and r_R have maxima at 9 and 19°C respectively

Reference

Haugen P and O Sten-Knudsen *J gen Physiol* 1976, *68* 247–265

C 6

rcomere Behaviour during Isometric Relaxation in Isolated Striated Muscle Fibres

By K. A. P Edman and F W Flitney Department of Pharmacology University of Lund, Sweden

emitendinosus muscle fibres of *R temporaria* were stimulated to produce fused isometric tani (plate electrodes, 0.2 ms square pulses 0–20°C) and the changes in sarcomere ngth were followed by streak photography of the diffraction spectra produced by illuminating the fibre with a laser (He Ne 1 mm beam width). Streak recordings were made t successive 1 mm intervals along the entire length of the fibres.

The sarcomeres in some segments were seen to shorten during relaxation, at the ex ense of those in other regions which elongated (Edman and Flitney 1977 and for further eferences). In about 30% of the segments studied the first order line fragmented into everal discrete maxima, and the individual microstriations were often observed to cross over revealing a tendency for sarcomeres having lengths marginally greater than the mean to shorten, and *vice versa*. The pattern of sarcomere movements remained constant in any one fibre. Regions which elongated were found at all possible locations but occurred more frequently near the fibre ends. The onset of the changes in length coincided with the 'shoulder' on the tension record, and the maximum displacement occurred at a time when tension approached zero (about 2% of maximum tetanic force at 2.20 μm sarcomere length).

The peak amplitude of the sarcomere elongation (ΔS_e) exceeded the amplitude of sarcomere shortening (ΔS_s) by an amount which depended upon the fraction of the fibre which shortened (0.71 ± 0.10 mean $\pm$ S.D) and the accompanying change in length of the series elastic element (2.01 ± 1.38% fibre length). Both ΔS_s and ΔS_e, and the mean velocity of the length change, decreased with increasing sarcomere length (range: 1.9–3.2 μm)

The pattern of length changes suggest that the duration of mechanical activity differs along the length of the fibre, being shortest in those segments which elongate during relaxation. A possible explanation for this is that the amount of calcium released, and/or the rate at which it is re-sequestered varies in different fibre segments. As a result some segments will remain active over a longer time and undergo further shortening during the tension decay stretching weaker regions which ultimately yield (at the 'shoulder' of the tension record) when the force per cross-bridge exceeds a critical value.

Reference

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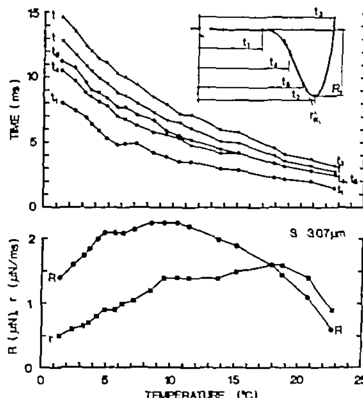


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C 8

The Timing of Muscular Contraction in Monkeys Landing from a Leap

By A. Mosfeldt Laursen P Dyhre Poulsen H Jahnsen and A. Djorup *Institute of Neurophysiology University of Copenhagen Denmark*

Muscular activity begins before the foot touches the ground in both stepping and sudden falls The activity absorbing the impact of landing, thus, is not elicited by impulses from receptors in the leg. To investigate the mechanism controlling the onset of anticipatory muscular activity we have studied the electromyogram of extensors and flexors in monkeys jumping from different heights

Forces on the landing platform and acceleration of the head were both maximal 10–15 msec after the fingers touched the platform. The acceleration of the head associated with landing was brief confined to about 10 msec and it was a linear function of the height of the jump (3 g/m). At the moment of peak acceleration the arms were in nearly full extension and during the subsequent flexion of the arms acceleration of the head was absent. A second peak one fourth the size of the first, occurred after about 55 msec when the head rose toward the position associated with normal posture

Electrical activity in the triceps began 44 msec before landing. The initial extensor burst lasted until about 19 msec after contact with the landing platform and subsequently 3 or 4 bursts each lasting 12 msec were separated by silent periods after about 10 msec Electrical activity in the biceps began 32 msec before the hands touched the platform but the burst ended 8 msec before contact. Subsequently extensor and flexor activity occurred in a reciprocal pattern.

The time of onset and the pattern of muscular activity were independent of the height of the jump. The onset thus occurred at greater heights the higher the jump The probability that the monkey turned the extensor on a constant time after departure from the starting platform was calculated from linear regression analysis and found to be less than 0 001

We suggest that most or all of the motor units in the extensor fire in synchrony to stiffen the arm, as they *did* during a forceful ballistic contraction studied by Desmedt and Godaux (1977) and that the activity in the flexor ceases by reciprocal inhibition when most or all of the motor units in the extensor fire in synchrony

C 9

Adrenoceptor Mechanisms in the Control of Smooth Muscle Activity in the Human Oviduct

By B Lindblom, L. Hamberger and B Ljung. *Department of Physiology and Obstetrics & Gynecology University of Göteborg Sweden*

Transport of the ovum through the human oviduct is assumed to be influenced physiologically by both hormonal and neuronal factors A muscular "tube-locking" mechanism under adrenergic control has been shown to exist in the ampullary-isthmic junction of the rabbit oviduct (Brundin 1964 Howe and Black 1973) and certain observations (Sjöberg 1967

C 7

Phase Dependent Modulation of the Transmission in Reflex Pathways during Fictive Locomotion

By O ANDERSSON H FORSSBERG and M LINDQUIST *Department of Physiology III Karolinska Institutet Stockholm Sweden*

In walking spinalized cats a tactile stimulation of the dorsum of the paw evokes a flexor reaction during the swing phase but an increased extensor activity during the support phase (Forssberg *et al* 1975 1977) The aim of the present investigation has been to analyze the neural mechanisms of this "phase dependent reflex reversal" To summarize three different models could contribute to the reversal 1 The cyclical changes in the excitability level of the motoneurone that occur concomitant with the locomotor commands" 2. A phasic depression or facilitation of the transmission in the reflex pathways to flexors and extensors *a*) by interaction from other peripheral pathways or *b*) by influence from the spinal "locomotor generator" responsible for the basic locomotor movements (see Forssberg *et al* 1977)

A preparation with "fictive locomotion" was used Adult cats were acutely spinalized paralyzed (tubocurarin) and injected i v by DOPA and Nialamid drugs which activate the "locomotor generator" (Grillner and Zanger 1974) The alternating locomotor activity was recorded from extensor and flexor muscle nerve filaments.

A weak electrical stimulation of the paw (? mA 0.5 ms) evoked large responses in the nerve filaments. During flexion there was large flexor responses and during extension large extensor responses Hence the "reversal" present in "walking immobilized animals" eliminated the possibility of phasic peripheral interaction (2*a*) as exclusive origin for the modulation.

By intracellular recordings from motoneurones it was possible to distinguish between the two remaining models In flexor motoneurones the paw stimulation evoked an EPSP after about 10 ms in some neurones followed by an IPSP In 16 of 20 motoneurones the EPSP was larger during flexion than during extension and in 8 of these significantly larger ($p<0.05$ T-test) Generally in the extensor motoneurones the size of the responses was smaller and the modulating effect less pronounced than in the flexor motoneurones

The results show that there is a phasic modulation of the transmission in different reflex pathways during "fictive locomotion" The effectiveness of different reflex pathways is thus controlled by the "spinal locomotor generator"—a phasic central gain control has been demonstrated

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i the amine level remains constant until the last two weeks during which it falls to the : low values as in the contralateral fetus-containing horn (Owman *et al* 1975).

the present investigation the mechanical responses to electrical field stimulation : studied in isolated myometrial strips from virgin guinea pigs pre-treated with estradiol ith estradiol and 6-OH-dopamine and from pregnant guinea pigs The uterine strips : contracted either spontaneously or by adding methacholine or bradykinine to the n bath. The adrenergic innervation of the uterus was also studied histochemically the noradrenaline content measured fluorometrically

lectrical stimulation caused a relaxation of the uterus from non-pregnant guinea pigs cing the tone about 30% Using hexamethonium, guanethidine, tetrodotoxin and ranolol it was shown that the responses were due to activation of postganglionic nergic nerves acting on β-adrenoceptors. Myometrial strips taken from pregnant ea pigs about two weeks before term and strips from animals pretreated with 6-OH amine showed no response to the stimulation. Addition of noradrenaline caused relaxa- of both the pregnant and the non-pregnant uterus mounted in the same organ bath. : threshold doses were lower and the responses greater in the pregnant uterus possibly cating a supersensitivity to noradrenaline When using animals with unilateral preg- cy the strips from the empty uterine horn were relaxed by electrical stimulation, while strips from the contralateral fetus-containing horn mounted in the same bath showed response.

n the non-pregnant guinea pigs there was a rich occurrence of adrenergic nerves in the ometrium, while no adrenergic uterine nerves could be seen in the pregnant animals or er treatment with 6-OH-dopamine The tissue content of noradrenaline was reduced the treatment with 6-OH-dopamine to about 5% of controls

t is concluded that the morphological disappearance of adrenergic nerve fibres and the uction of transmitter content in the guinea pig uterus during pregnancy is accompanied a loss of adrenergic nerve function possibly resulting in a denervation supersensitivity the transmitter

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C 11

...cle Metabolites during Recovery after Maximal Exercise in Man

By O VAAGE, E. NEWSHOLME O GRØNNERØD and L. HERMANSEN *Institute for Work Physiology Oslo Norway*

In a recent study it was shown that only a small fraction of the lactate produced in skeletal muscle during maximal exercise disappeared from muscle into circulation during recovery ... observation and our findings of a rapid lactate disappearance in muscle, a rapid

Owman *et al* 1967 1976) give evidence for a similar arrangement in the human oviductal isthmus The timing of ovum transfer from the ampulla of the oviduct to the uterus might thus be regulated by the degree of activation of excitatory and inhibitory adrenoceptor mediated mechanisms in the smooth muscle of the tubal wall. To classify these receptors in the alfa- and beta types small strips from the circular (inner) and longitudinal (outer) smooth muscle layers in the isthmic part of human Fallopian tubes were used for studies of isometric contractile activity in organ baths.

Addition of adrenaline (A) or noradrenaline (NA) at a concentration of 1–10 μM resulted in a marked increase in the spontaneous phasic activity of the longitudinal specimens, while circular preparations consistently were inhibited The inhibitory response to A and NA was reversed into a slight increase in activity after beta-blockade with propranolol (30 μM) The same type of response was elicited after addition of an alfa-adrenergic agonist phenylephrine (1–30 μM) Isoprenaline (1–100nM) markedly inhibited the contractility in both circular and longitudinal preparations

The beta$_2$-agonist terbutaline caused no alteration of contractility in concentrations lower than 0.3 μM With higher concentrations of terbutaline (0.3–10 μM) a gradually diminished activity was obtained in both circular and longitudinal specimens.

It is concluded that the adrenoceptors in the circular muscle layer of the human oviduct are predominantly inhibitory of the beta-type In the longitudinal muscle layer the receptors are mainly of the alfa-type This differentiation in distribution of alfa- and beta-receptors in the two muscle layers may play a functional role in the physiological control of ovum transport

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C 10

Disappearance of Adrenergic Innervation in the Guinea Pig Uterus during Pregnancy

By M ELMÉR P ALM and G THORBERT *Institutes of Physiology Histology and Obstetrics and Gynecology University of Lund Sweden*

The smooth musculature in the uterus of the guinea pig is innervated by adrenergic nerves and the content of neuronal noradrenaline measured fluorometrically varies during pregnancy and under the influence of sex hormones (Sjöberg 1968) In animals with unilateral pregnancy the content of noradrenaline in the pregnant uterine horn is continuously re- ... eeks of gestation to reach near zero levels at term In the empty

C 12

...al Medullary Control of Muscular Glycogenolysis and Pancreatic Hormonal Secretion ...g Exercise in Rats

By E. A. RICHTER, B SONNE, J J HOLST and H. GALBO. *Institute of Medical Physiology B and Institute of Medical Physiology C University of Copenhagen Denmark*

...have previously reported (Galbo *et al* 1977) that in adrenodemedullated rats which ...been injected with 6-hydroxydopamine a drug that selectively destroys sympathetic ...endings (Thoenen and Tranzer 1968) muscular glycogen depletion as well as gluca... ...secretion are decreased during prolonged exercise whereas insulin secretion is in... ...sed compared to the findings in control rats. In the present study we have elucidated ...ese effects of sympathectomy could be ascribed selectively to the lack of either the ...al medulla or of the peripheral sympathetic nerve endings

...roups of eight rats were either adrenal demedullated (DM) injected with 6-hydroxy ...mine (6-OHD) adrenal demedullated and injected with 6-hydroxydopamine (SX) or ...operated and sham injected controls (C). The rats either rested or were forced to ...with a tail weight (2% of body weight) for 100 min in tepid water Subsequently ...d was drawn by cardiac puncture and samples from the soleus and the deep part of ...vastus lateralis muscle were collected

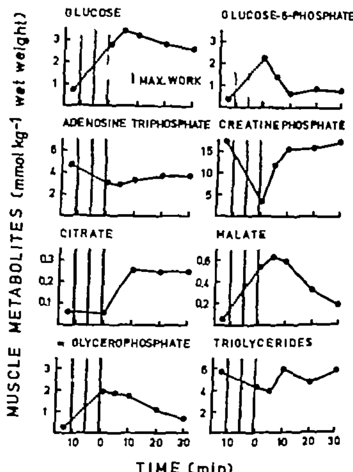
...uring exercise the plasma concentrations of insulin were higher and plasma glucagon ...lactate concentrations were lower in SX and DM-rats compared to C and 6-OHD- ... Blood glucose concentrations increased only in 6-OHD (25%) and C-rats (61%). ...ing exercise muscle glycogen concentrations in the soleus muscle decreased 50% and ...in 6-OHD- and C-rats respectively (P<0.05) In the deep part of the vastus lateralis ...cle the decreases in glycogen concentrations were 54% and 41% respectively (P< ...). However in DM and SX-rats the markedly reduced decreases were: 9% and 0% ...ectively (P>0.05) in the soleus muscle and 26% (P<0.05) and 9% (P>0.05) respec... ...y in the deep part of the vastus lateralis muscle.

...is concluded that in exercising rats the adrenal medullary hormones significantly ...ance muscular glycogen depletion as well as pancreatic α-cell secretion and significant ...inhibits β-cell secretion. The peripheral sympathetic nerves however only play a ...or role in the control of muscular glycogen depletion and pancreatic hormonal secre... ...during exercise.

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muscle glycogen resynthesis and almost no uptake of glucose by the muscles during re covery were offered in support of Meyerhof's original hypothesis of a conversion of lactate into glycogen in skeletal muscle To further test this hypothesis changes in the concentrations of adenosine triphosphate (ATP) creatine phosphate (CP) triglycerides and different glycolytic intermediates were measured in the quadriceps muscle of 15 healthy male subjects at rest before intermittent maximal exercise and at different time intervals during the recovery period (30 min) The results (Fig 1) indicated that changes in the glycolytic intermediates could account for only a small fraction of the glycogen synthesized during the recovery period The glucose and glucose-6-phosphate concentrations during recovery are consistent with an inhibition of hexokinase and the small uptake of glucose by muscle reported earlier Moreover the concentrations of ATP CP and citrate observed during recovery suggest that phosphofructokinase is also inhibited Altogether the results of the present investigation are consistent with the hypothesis of a conversion of lactate into glycogen in human skeletal muscle

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C 14

[Indepe]ndence of Chronotropic and Inotropic Effects on Stroke Volume Regulation in the Dog

[O.] ILEBEKK, J LEKVEN and F KIIL *University of Oslo Institute for Experimental [M]edical Research Ulleval Hospital Oslo Norway*

[Usuall]y a rise in heart rate is combined with increased sympathetic activity The present [stud]y was undertaken to separate influences of changes in chronotropy and inotropy on [left ven]tricular stroke volume

[Hear]t rate was increased in steps by right atrial pacing in open-chest dogs at control [inotrop]y during raised inotropy by continuous isoproterenol infusion (0.20–0.25 μg/kg [...] and during reduced inotropy by propranolol administration (0.5 mg/kg) Left [v]entric[ular di]mensions were studied by continuously recording myocardial chord lengths (MCL) [betwe]en ultrasonic elements sewn into the anterior wall of the left ventricle (Bugge-[Asper]heim *et al.* 1969)

[At an]y level of inotropy stroke volume declined linearly to increments in heart rate [At cons]tant heart rate a rise in inotropy increased stroke volume and stroke volume de[clined] after propranolol administration. The difference in stroke volume at the various [levels] of inotropy was not influenced by heart rate

[Left] ventricular end-diastolic MCL, an estimate of end-diastolic left ventricular volume [was onl]y a function of heart rate and not of inotropy End-diastolic MCL declined linearly [to incre]ments in heart rate with the same slope at any level of inotropy Changes in ino[tropy] at constant heart rate did not influence end-diastolic MCL, except at very high heart [rate w]hen end-diastolic MCL was slightly higher during isoproterenol infusion than at [contro]l inotropy

[End]-systolic MCL, an estimate of end-systolic left ventricular volume varied greatly [with] changes in inotropy so that a difference in end-systolic volume could account for the [change i]n stroke volume. End-systolic MCL decreased only slightly as heart rate was in[crease]d almost equally at any level of inotropy Isoproterenol reduced end-systolic MCL [and in]creased stroke volume whereas propranolol had the opposite effect at constant [heart] rate.

[In] conclusion, chronotropic changes mainly influence end-diastolic left ventricular di[mensi]ons, whereas inotropic changes mainly influence end-systolic dimensions. Chrono[tropy] and inotropy therefore affect stroke volume regulation through virtually independent [mecha]nisms.

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C 13

Left Ventricular Hypertrophy in Spontaneously Hypertensive Rats (SHR)—Consequences of and Compensation for the Altered Frank-Starling Relationship

By E NORESSON S E. RICKSTEN M HALLBÄCK and P THORÉN *Department of Physiology University of Göteborg Sweden*

The degree of left ventricular hypertrophy in hypertension is mainly a consequence of raised afterload but the functional importance has been subjected to considerable debate. The common decrease of stroke volume with progressing hypertension and cardiac hypertrophy may suggest impaired myocardial performance Further in isolated perfused SHR hearts the Frank-Starling curve is displaced to the right of that of controls (NCR) However this displacement appears to be a consequence of reduced diastolic compliance, leading to a lower degree of myocardial prestretch in SHR for a given filling pressure (Hallbäck *et al* 1975) The maintenance of equal stroke volumes in SHR and NCR *in vivo* would then call for either increased inotropism or/and raised filling pressure in SHR.

To further explore this situation left atrial catheters were chronically implanted in 7 pair of matched NCR and SHR. Left atrial pressure was measured in the end-expiratory phase being largely twice as high in SHR as in NCR (10.3±0 4 mm Hg ±0 3 mm Hg and 4 6±0.3 mm Hg respectively p<0 001)

For additional analysis of the hemodynamics during left ventricular hypertrophy the Frank-Starling relationship was studied at various levels of constant afterload in 17 pairs of SHR and NCR hearts The paced hearts were perfused *in vitro* in an antegrade perfusion system Preload and afterload could be independently varied during recordings of cardiac output. At equal afterloads below 100 mm Hg the SHR Frank Starling curve was displaced to the right of the NCR one at physiological" preloads and maximal stroke volumes were largely the same With higher afterloads the SHR hearts maintained their maximal stroke volume considerably better than the NCR ones However the SHR curves were still displaced to the right of the NCR ones in the lower preload range though crossing over at higher filling pressures

Thus the hypertrophic adaptation of the SHR heart has at least two important hemodynamic consequences At high afterloads it is certainly able to better maintain stroke volume than the NCR heart provided the end-diastolic pressure is high enough for optimal prestretch Nevertheless due to the reduced diastolic compliance of the thicker walls the SHR left ventricle *in vivo* can in the *ordinary* range of filling pressure maintain the same stroke volume as NCR only if the filling pressure is kept higher or/and inotropism is enhanced

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C 14

...ndence of Chronotropic and Inotropic Effects on Stroke Volume Regulation in the Dog

L. ILEBEKK, J. LEKVEN and F. KIIL, *University of Oslo, Institute for Experimental Medical Research, Ullevaal Hospital, Oslo, Norway*

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C 15

Mechanisms Involved in Transcapillary Fluid Movement into the Secreting Cat Submandibular Gland

By J LUNDVALL and J HOLMBERG *Department of Physiology and Biophysics University of Lund Sweden*

An attempt was made to elucidate the mechanisms involved in the process of net transcapillary fluid movement from the intravascular to the extravascular space of the secreting submandibular gland of the cat. The capillary hydrodynamic conductivity (CFC) in the resting gland was found to be 0.30–0.35 ml/min×100 g tissue×mm Hg and it increased upon parasympathetic stimulation (20 Hz) in rough relation to the nerve excitation rate to a maximal value of about 1 0 ml/min×100 g×mm Hg. Comparison of the CFC in states of maximal vasodilatation evoked by chorda stimulation and by papaverine infusion indicated that the increase of CFC during parasympathetic activation entirely could be ascribed to an increase of the functional capillary surface area available for transcapillary fluid exchange whereas the specific capillary permeability remained constant. Capillary hydrostatic pressure (Pc) in the resting gland was about 20 mm Hg and increased by 18 mm Hg on the average during maximal vasodilatation produced by chorda activation. Roughly 65% of the total transcapillary fluid transfer from blood into the secreting gland could be ascribed to filtration caused by the increase of Pc. It is suggested that the remaining 35% of the fluid transfer was caused by osmosis resulting from regional tissue hyperosmolality in the active gland The tissue hyperosmolality as traced in the venous effluent, averaged 18 mOsm/kg H O at 20 Hz and was found mainly due to increased concentrations of sodium and lactate

C 16

Secretory Potentials Evoked by Sympathetic Stimulation of the Parotid Gland of Cat

By P THESLEFF N EMMELIN and W GRAMPP *Department of Physiology and Biophysics University of Lund Sweden*

Ekström and Emmelin (1974) have demonstrated that sympathetic stimulation of the parotid gland of cat gives rise to a β-mediated salivary secretion The corresponding electrophysiological events in the secretory cells were however largely unknown To investigate these cats were anaesthesized with chloralose their parotid glands exposed and cells down to a depth of 1 0–1.5 mm impaled by KCl or K citrate-filled bevelled microelectrodes with resistances ranging between 10 and 15 megohms Recorded signals were processed using conventional electrophysiological techniques For stimulation of the parotid gland supramaximal shocks as judged by pupillary dilatation or salivary secretion were applied to the sympathetic trunk or auriculo-temporal nerve

In the great majority of cells impaled by either KCl or K citrate-filled electrodes stimulation of the sympathetic trunk gave rise to a membrane depolarisation which was ac

...mpanied by a marked decrease in membrane resistance. Both the latency rise time and ...plitude of this depolarisation were dependent on stimulation frequency It thus became ...ble at liminal frequencies of 0 1–0.2 Hz and then grew with increasing frequency reach... ..., at about 5 Hz, a maximal size of about 20 mV in cells with a normal resting potential 35–40 mV At the same time the latency and rise time of the depolarisation were short... ...ed from several tens of seconds to 1–3 s and 5–8 s, respectively These times could be ...ther reduced by additional increases in stimulation frequency At moderate stimulation ...quencies the depolarisation was maintained throughout stimulation and at supramaximal ...mulation frequencies it outlasted stimulation by several seconds before declining grad...ally

Both depolarisation and decrease in membrane resistance due to sympathetic stimula...n were abolished or greatly reduced by i.v injection of practolol (2 mg/kg) Neither ...f these effects was however influenced by dihydroergotamine (0.5–1.0 mg/kg) or by ...ropine (200 μg/kg) which was able to completely abolish short latency hyperpolarising ...cretory potentials evoked by auriculo-temporal nerve stimulation.

As an additional observation it was noted that in part of the cells investigated the de...olarisation due to sympathetic stimulation was preceded by a short latency hyperpolaris...ng transient which was abolishable by atropine.

In conclusion, sympathetic stimulation gives rise to a β-mediated depolarisation which ...resumably results from altered ionic permeabilities of the membrane Also there seems ...o be some interaction between sympathetic and parasympathetic innervation which may ...e of importance in the control of the gland's secretory activity

Reference

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C 17

Inhibition of Gastric HCO Secretion by Noradrenaline and Adrenaline

By Gunnar Flemström, *Department of Physiology and Medical Biophysics University of Upp al Sweden*

Active secretion of HCO_3 by the surface epithelial cells was recently demonstrated in gastric antral and fundic mucosa *in vitro* The rate of this secretion is approximately 10 percent of maximal H secretion in the same species It is stimulated by carbamylcholine db-cGMP and Ca^{++} but not affected by histamine gastrin or db-cAMP and shows higher sensitivity than H secretion to acetazolamide. A very similar gastric secretion of HCO_3^- has been demonstrated also in the guinea pig *in vivo*

The function of gastric HCO_3^- secretion may be to reduce H concentration in the immediate vicinity of the luminal surface of the gastric epithelium and thus act as a protective mechanism against acid. Acetazolamide which inhibits HCO_3^- secretion also decreases

C 15

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companied by a marked decrease in membrane resistance. Both the latency rise time and amplitude of this depolarisation were dependent on stimulation frequency It thus became visible at liminal frequencies of 0 1–0.2 Hz and then grew with increasing frequency reaching at about 5 Hz, a maximal size of about 20 mV in cells with a normal resting potential of 35–40 mV At the same time the latency and rise time of the depolarisation were shortened from several tens of seconds to 1–3 s and 5–8 s respectively These times could be further reduced by additional increases in stimulation frequency At moderate stimulation frequencies the depolarisation was maintained throughout stimulation and at supramaximal stimulation frequencies it outlasted stimulation by several seconds before declining gradually

Both depolarisation and decrease in membrane resistance due to sympathetic stimulation were abolished or greatly reduced by i.v injection of practolol (2 mg/kg) Neither of these effects was however influenced by dihydroergotamine (0.5–1 0 mg/kg) or by atropine (200 μg/kg) which was able to completely abolish short latency hyperpolarising secretory potentials evoked by auriculo-temporal nerve stimulation.

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In conclusion, sympathetic stimulation gives rise to a β-mediated depolarisation which presumably results from altered ionic permeabilities of the membrane Also there seems to be some interaction between sympathetic and parasympathetic innervation which may be of importance in the control of the gland's secretory activity

Reference

LUNDBERG, J and N EMMELIN *Quart J exp Physiol.* 1974 59 11–17

C 17

Inhibition of Gastric HCO^- Secretion by Noradrenaline and Adrenaline

By GUNNAR FLEMSTRÖM. *Department of Physiology and Medical Biophysics University of Uppsala S eden*

Active secretion of HCO by the surface epithelial cells was recently demonstrated in gastric antral and fundic mucosa *in vitro*. The rate of this secretion is approximately 10 percent of maximal H secretion in the same species. It is stimulated by carbamylcholine, 8-cGMP and Ca^{++} but not affected by histamine gastrin or db-cAMP and shows higher sensitivity than H secretion to acetazolamide A very similar gastric secretion of HCO_3^- has been demonstrated also in the guinea pig *in vivo*

The function of gastric HCO_3^- secretion may be to reduce H concentration in the immediate vicinity of the luminal surface of the gastric epithelium and thus act as a protective mechanism against acid. Acetazolamide which inhibits HCO_3^- secretion also decreases

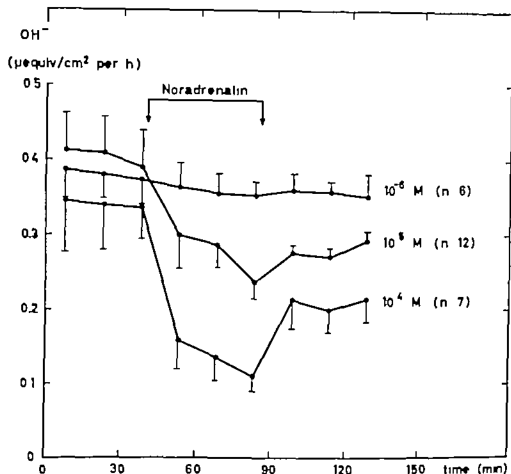


Fig. 1 Effects of some concentrations of noradrenaline on luminal alkalinization in the *Rana temporaria* fundus. Means ±S E are given None of the concentrations caused significant changes in transtissue electrical resistance or potential difference The histamine H receptor antagonist Metiamide (10^{-4} M) was used throughout the experiments to inhibit H secretion.

the ability of the gastric epithelium to resist luminal acid This made it of interest to study the effects of some catecholamines on gastric HCO_3 secretion

Frog (*Rana temporaria*) or *Necturus* fundic or antral mucosae were mounted in *in vitro* chambers Antrum secreted HCO_3^- spontaneously while histamine H receptor antagonists were used in fundus to inhibit H secretion and thus reveal alkalinization

Noradrenaline (10^{-4} M) inhibited HCO_3 secretion both in fundus (Fig 1) and in antrum. Adrenaline had a similar effect but a higher concentration (10^{-4} M) was required while isoprenaline (10^{-3} M) was without effect. Pretreatment with phentolamine prevented the effect of noradrenaline At the concentrations used here the catecholamines did not affect gastric H^+ secretion

The results suggest that the inhibitory effect of catecholamines on HCO_3 secretion is mediated via *alfa* receptors Sympathetic activation may decrease the ability of the gastric epithelium to resist acid but leave the production of acid intact.

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C 18

…endence of Salivary Secretion, Passive and Active Potassium Transport on Flow Rate in … Perfused Submandibular Gland

By L. P Laugesen J O D Nielsen and J Hedemark Poulsen *Institute of Medical Physiology Dept A University of Copenhagen The Panum Institute Blegdamsvej 3 C DK-2200 Copenhagen N Denmark*

…-perfused salivary glands have been used as models to study electrolyte and water …sport in exocrine glands. However it appears that while the secretory rate in response …supramaximal stimulation is very similar during autoperfusion as compared to perfusion …h Locke solution (Poulsen 1975) the rates of the stimulation-induced passive loss of …racellular potassium to the perfusate as well as the post-stimulatory active reuptake of …assium are substantially *higher* during artificial perfusion than during autoperfusion …rgen 1956; Laugesen, Nielsen & Poulsen 1976) To establish whether these differ…ces could be due to the difference between the high flow of saline in the artificially per…sed gland and the much lower flow of plasma water in the autoperfused glands, we have …died the effect of the flow rate on secretory rate passive potassium loss and active …assium reuptake.

Cat submandibular glands were perfused with phosphate-buffered, oxygen saturated …cke solution at 37°C. The glands were stimulated with acetylcholine (10^{-4} M) for 90 s. The flow of perfusion fluid was varied by changing the arterial hydrostatic pressure be…een 90 and 50 mmHg. The potassium concentration of the venous effluent was measured …y flame photometry and the secretory rate was determined gravimetrically

The secretory rate was found to be largely independent on the flow of venous perfusion …id in the range 5–17 ml/min. By contrast the rate of the passive stimulation-induced …ss of intracellular potassium increased proportionally to the flow in the range 3–8 ml/…n, while a further increase in flow only caused a very moderate increase in the rate of …e potassium loss. Surprisingly the rate of the active post stimulatory reuptake of po…tassium from the perfusion fluid increased proportionally to the flow within the whole …nge studied (3–18 ml/min).

It is concluded that the very high rates of passive and active potassium transport be…tween salivary gland cells and perfusion fluid observed during artificial perfusion can be …scribed to the unphysiologically high vascular flow rate of water and electrolytes. The

experiments also demonstrate that the active transport mechanism responsible for the post stimulatory reuptake of potassium is very far from being saturated under physiological conditions

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C 19

Functional Interaction between Fish Hemoglobin Erythrocytic Nucleoside Triphosphates and Magnesium

By ROY E. WEBER *Department of Zoophysiology University of Aarhus*

Recent studies (Wood & Johansen 1972 Geoghegan & Poluhowich 1974 Weber *et al* 1975) have shown that the nucleoside triphosphates (NTP) ATP and GTP (guanosine triphosphate) are allosteric effectors that continuously adapt the O_2 affinity of hemoglobin

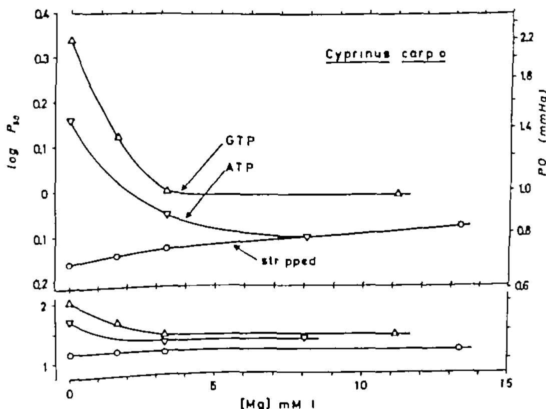


Fig. 1 The effect of magnesium ion on the half-saturation O_2 tension P_{50} and Hill's coefficient N of "stripped" (phosphate-free) carp hemoglobin (O) and hemoglobin solutions containing 0 16 mM ATP (∇) and GTP (Δ) measured at 20°C in Tris buffer pH 7 55 Hemoglobin concentration, 0 10 mM total chloride 0.10 mM

n fish erythrocytes to environmental conditions such as water hypoxia. As with diphosphoglycerate in mammals, these polyanionic cofactors bind at a specific positively charged site of deoxyhemoglobin depressing its O_2 affinity. Erythrocytes however contain relatively high concentrations of Mg^{2+} which will complex with NTP's impairing their role as regulators of erythrocytic O_2 affinity. This communication reports the effects of ATP and GTP on the O_2-binding properties of carp hemoglobin and the influence on this of Mg^{2+} in attempts to assess the physiological implications of the hemoglobin-ATP/GTP-Mg^{2+} interaction in fish.

ATP and GTP strongly increase the half-saturation O_2 tension (P_{50}) of the phosphate-free hemoglobin (depress O_2 affinity) and raise the Hill coefficient n, which reflects the sigmoid character of the O_2 equilibrium curve (cf. Fig. 1). However at equal concentrations the GTP effects markedly exceed those of ATP. Double logarithmic plots of *log* P_{50} v phosphate concentration indicate that binding of both NTP's liberates all bound O_2 from carp oxyhemoglobin.

Mg^{2+} drastically reduces the effects of NTP on O_2 affinity (Fig. 1). However while increasing Mg^{2+} obliterates the ATP effect, a GTP effect persists. This correlates with previous evidence that GTP binds at an additional site of the hemoglobin perhaps through its greater ability for hydrogen binding (Weber *et al.* 1975) and indicates that the Mg-GTP complex itself inhibits O_2 binding. Hypoxic- and normoxic-acclimated carps show no significant difference in erythrocytic Mg^{2+} concentrations indicating that the adaptational changes in blood P_{50} are primarily attributable to variation in NTP levels.

The data demonstrate a dominant role for GTP in the *in vivo* regulation of blood P_{50} in fish containing both types of trinucleotides.

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C 20

Influence of EMG and Tension on the Cardiovascular Response to Isometric Muscle Contractions

By B. SCHIBYE, J. H. MITCHELL, B. SALTIN and F. C. PAYNE. *August Krogh Institute University of Copenhagen, Copenhagen, Denmark*

It is still an open question to what degree the regulation of the cardiovascular system during isometric muscle contractions is of central and/or peripheral origin. The aim of the present experiments was to investigate the possible effect of central nervous irradiation and peripheral muscle tension on heart rate (HR) and blood pressure (BP) during sustained isometric contraction of the quadriceps muscle, the EMG of vastus lateralis taken as a measure of the [illegible]l nervous activity. Seven male subjects went through two different

experiments also demonstrate that the active transport mechanism responsible for th post stimulatory reuptake of potassium is very far from being saturated under physiolog cal conditions

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C 19

Functional Interaction between Fish Hemoglobin Erythrocytic Nucleoside Triphosphate and Magnesium

By ROY E WEBER *Department of Zoophysiology University of Aarhus*

Recent studies (Wood & Johansen 1972 Geoghegan & Polubowich 1974 Weber *et al* 1975) have shown that the nucleoside triphosphates (NTP) ATP and GTP (guanosine triphosphate) are allosteric effectors that continuously adapt the O_2 affinity of hemoglobin

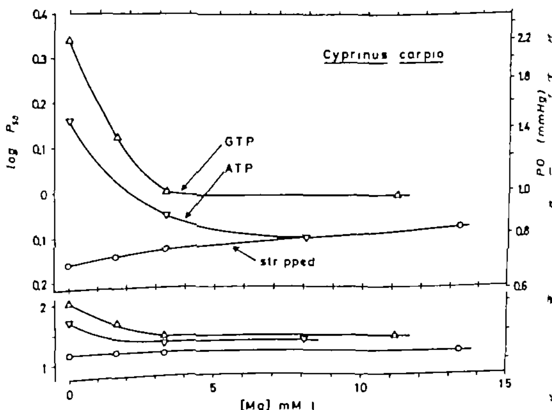


Fig. 1 The effect of magnesium ion on the half-saturation O_2 tension, P_{50}, and Hill's coefficient N of "stripped" (phosphate-free) carp hemoglobin (O) and hemoglobin solutions containing 0 16 mM ATP (∇) and GTP (Δ) measured at 20°C in Tris buffer pH 7.55 Hemoglobin concentration 0 10 mM total chloride 0 10 mM

arked increases of the plasma VIP concentration were noted in the venous effluents om the target organs. No change of arterial VIP concentrations was observed. Further ore, electrical stimulation of the vagal *low threshold* fibres, evoking atropin sensitive astric contractions, produced no increase in gastric venous plasma concentration of VIP. The results obtained thus suggest that VIP may be a neurotransmitter in the gastrointestinal tract mediating specific physiological reflexes and constituting a nervous control system similar to the established cholinergic and adrenergic ones.

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C 22

Effects of Centrally Infused Histamine (HA) and its Analogues in the Conscious Goat

By L. Eriksson and L. Tuomisto. *Department of Physiology, College of Veterinary Medicine, Helsinki and Department of Pharmacology, University of Helsinki, Finland*

Histamine (HA), a putative neurotransmitter, is richly located in the hypothalamus (*cf.* Schwartz 1977). In the guinea pig hypothalamus the increases in the HA content and in the activity of HA-N-methyltransferase occur approximately at the same time during neurogenesis (Tuomisto 1977) as does the appearance of vasopressin-containing neurosecretory granules (Donev 1970, Silverman 1975). Therefore the effects of intracerebroventricularly (ICV) infused HA on the water balance are of great interest. In this study HA and its analogues were given ICV to conscious hydrated goats.

Infusion of HA into the third or lateral brain ventricles (10–1 000 μg/animal) caused a dose-dependent antidiuretic response in the hydrated goat. The urine osmolality began to rise within 20 min, reached its maximum within 40–130 min and lasted for 90–290 min depending on the dose.

2-Methylhistamine (2-MH), an H_1-agonist, caused a weaker but otherwise similar antidiuretic response as HA. Dimaprit (300 μg), a specific H_2-agonist, did not change urine osmolality appreciably during the first 3 hours. Subsequently, however, the osmolality was increased. 4-Methylhistamine (4-MH), another H_2-agonist with some H_1-activity, given at a dose of 300 μg, increased the urine osmolality gradually within a 3-hour period. 1 mg of the same agent primarily induced a HA-like antidiuresis. After a partial recovery a secondary bout of antidiuresis ensued.

1-Methylhistamine, a metabolite of HA, did not influence the urine osmolality.

Other effects of the ICV infusions of HA and its analogues were slight drowsiness, except after 1-MH, and an elevation of rectal temperature after the H_2-agonists.

procedures 1) Twenty percent of the maximal voluntary isometric force (%MVC) was maintained for 5 min while HR BP and EMG was followed continuously 2) As experiment 1) except that the EMG was voluntarily kept constant at an initial 20% MVC-level In 1) a continuous rise was seen in HR BP and EMG throughout the 5 min (mean values from 0 to 5 min HR 77 6 to 108 0 BP 130.2/83 4 to 180.3/122 6 mmHg, EMG 112.7 to 218 6 mV) In 2) an increase in HR and BP and a decrease in isometric tension was observed (mean values from 0 to 5 min HR 78 0 to 95 0 BP 133 1/84 9 to 154.3/103.3 mmHg tension 19.5 to 11 0%MVC) Looking at the 5 min values it is not possible to deduce whether it is the muscle tension or the EMG which is responsible for the changes in HR and BP since the percentage increase of HR and BP in 2) is about half the increase in 1) while both the EMG and tension in 2) is about half the value of that seen in 1) If however HR and BP is followed during the whole period it can be seen that both parameters increase in both experiments in spite of the fact that muscle tension is constant or decreasing. EMG rose continuously in 1) while it was kept constant in 2) Thus neither tension nor EMG is alone responsible for the changes in HR and BP One possible explanation may be deduced from the fact that most of the increase in HR and BP in 2) was seen during the first two min where tension was reduced 50% while a continuous increase in HR and BP was seen in 1) This may indicate that also local metabolic factors may be involved in the cardiovascular regulation during isometric exercise

C 21

Is Vasoactive Intestinal Polypeptide (VIP) a Neurotransmitter in the Gastrointestinal Tract?

By J FAHRENKRUG U HAGLUND M JODAL O LUNDGREN L. OLBE and O B SCHAFFALITZKY DE MUCKADELL, *Departments of Physiology and Surgery University of Göteborg Sweden and Department of Clinical Chemistry Bispebjerg Hospital Copenhagen Denmark*

The presence of VIP in central and peripheral neurons has been demonstrated with immunohistochemical techniques (Larsson *et al* 1976) mainly located in synaptosomes (Giachetti *et al* 1977) These findings suggest that VIP may be a neurotransmitter Within the gastrointestinal tract the localization of the VIP containing neurons varies In the stomach they are mainly found in the muscularis layer while in the small and large intestines they are most abundant in the mucosa where they often follow the small blood vessels Except in the gastric antrum no VIP immunoreactivity was found in the epithelial cells of the alimentary canal To test the hypothesis of VIP as a neurotransmitter three different nervously controlled effects known to be mediated via non-cholinergic non-adrenergic mechanisms were studied in cats. 1 The gastric relaxation elicited by stimulating electrically the *high threshold* vagal fibres 2. The intestinal vasodilatation evoked by mechanically stimulating the intestinal mucosa. 3 The colonic vasodilatation observed upon electrical stimulation of the pelvic nerves The plasma concentration of VIP before during and after inducing the nervous effects were determined using the RIA method of Fahrenkrug and Schaffalitzky (1977) Concomitant with the nervously induced effects

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C 24

Role of Cyclic AMP in Oocyte Maturation

By C. Ekholm, T Hillensjö, C. Magnusson and K. Ahrén *Department of Physiology University of Göteborg Göteborg Sweden*

One of the consequences of the preovulatory surge of luteinizing hormone (LH) is resumption of oocyte meiosis ("oocyte maturation"). The mechanism behind this LH effect in the preovulatory follicle is still unknown. Cyclic AMP has been suggested to play a role in this mechanism since it has been shown (Tsafriri *et al* 1972) that injection of cyclic AMP into preovulatory rat follicles induced oocyte maturation. It has on the other hand, also been reported (Cho *et al* 1974) that dibutyryl cyclic AMP (dbcAMP) inhibited the spontaneous maturation of isolated mouse oocytes. The aim of the present study has been to further elucidate the possible role of cyclic AMP in this context.

Preovulatory follicles were obtained from immature rats pretreated with a low dose of pregnant mare's serum gonadotrophin two days earlier Oocytes were dissected from the follicles and oxygen consumption of the isolated oocytes was measured according to Hultben (1974). Respiration was measured in oocytes directly isolated from the follicles and in oocytes first cultured in a modified Brinster phosphate buffer (Magnusson *et al* 1977) prior to determination of oxygen consumption. The oocytes were examined by interference contrast microscopy using germinal vesicle breakdown (GVB) as the first sign of oocyte maturation. The cultured oocytes, which had resumed meiosis (all with GVB), showed higher oxygen consumption than the dictyate oocytes taken directly from the preovulatory follicles. When cyclic AMP (10 mM) or dbcAMP (1 mM) was added to the culture medium GVB was prevented and the rise in oxygen consumption was abolished. The effect of cyclic AMP was reversible since GVB was present after another 4 hrs culture period in nucleotide free medium.

In a second series of experiments isolated preovulatory follicles were treated in a double incubation procedure starting with 2 hrs incubation with dbcAMP (10 mM) followed, after a 30 min wash period by 4 hrs incubation with or without dbcAMP For one group of follicles plain medium was used for both incubation periods. The last group of follicles showed a low rate of GVB (17%) while presence of dbcAMP throughout the whole incubation period completely abolished GVB Transfer from dbcAMP-containing to plain medium, on the other hand, enabled 70% GVB The inhibiting effect of dbcAMP on oocyte maturation was thus not only reversible but dbcAMP if present only in the first incubation, exerted a stimulatory effect on GVB

A possible interpretation of our own data as well as other experiments is 1) that an increased level of cyclic AMP in or around the oocyte itself is not involved in the mechanism

These results confirm the earlier observation from this and other laboratories of the antidiuretic effect of HA in various species (*cf* Schwartz 1977) Our studies with H_1- and H_2-agonists speak for the role of H -receptors in the HA induced antidiuresis in the hy drated goat. The delayed antidiuretic effect of the H_2-agonists needs further study In part it may result from the increased body temperature

The gifts of histamine analogues from the Smith Kline and French Laboratories are gratefully acknowledged

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C 23

Dopamine-β-hydroxylase and Calcium in Adrenaline- and Noradrenaline-rich Granules Bovine Adrenal Medulla

By G BOLSTAD G SERCK HANSSEN and K B HELLE

The discharge of soluble dopamine-β-hydroxylase (DβH) activity in concert with cat cholamines (CA) has been taken as a measure of the exocytosis of the catecholamine ho mones in response to neurochemical and electrical stimulation of the adrenomedulla cells (Viveros Arqueros and Kirshner 1968) Acethylcholine (100 μM) stimulates how ever the release of less enzyme activity/μmole CA from the perfused bovine adren than apparent in the core of the isolated chromaffin granules (Helle and Serck Hanssen 1977) Furthermore stimulus related DβH activity correlates in a positive manner wit NA but not with A These observations might suggest exocytotic release from a sub population of granules with a composition different from that of the total population o chromaffin granules

The chromaffin granules can be partially separated into A and NA rich fractions by sedimentation in hypertonic gradients ranging from 1 6–2 0 M sucrose (Eade 1958) Gran ules have now been sedimented into 2 ? M sucrose These granules contained 66% o their amines as NA and were considerably richer in total amines (5 05±0 86 μmoles CA/ mg protein mean ±S E n=10) than the A rich granules sedimenting in the 1 6 M sucrose layer (69% A ? 44±0 29 μmoles CA/mg protein mean ±S E n=10)

The NA-rich granules are considerably lower in their ratio of soluble enzyme activity per μmoles CA than the A-rich granules and contain twice the amount of calcium per unit weight

It remains to be established to what extent these NA rich granules participate in the exocytotic release of the marker enzyme

R1-6 cells could have double-peaked spectral sensitivities and only the R7 single-d UV sensitivity

In this study we measured the spectral sensitivity of individual receptors and second order neurons in retina of fly *Calliphora*. Intracellular recordings were carried out with capillary microelectrodes filled with Procion Yellow solution. In total 43 receptors and 5 second order neurons were recorded and identified. Three groups of receptors found: cells with high UV (353 nm) and very low visible region sensitivity (1 0.03), with high UV and low visible region sensitivity (1 0.85) and cells with low UV but visible region sensitivity (0.65 1). Two major groups of second order neurons were found: cells with high UV and relatively low visible region sensitivity (1 0.35) and cells with high sensitivity in both regions (1 0.94). The sensitivity recordings were made in non-saturated region of the intensity-response function. The spectral sensitivity of the cell was not found to be attributed to the same morphological structure in different preparations. These preliminary results give a rise for assumption that a revision of morphological classification is necessary according to the cell function.

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C 26

Influence of Nerve–muscle Activity on the Elimination of Synapses in Neonatal Rat ... Muscle

By W. THOMPSON, D. KUFFLER and J. K. S. JANSEN. *From the Institute of Physiology, University of Oslo, Norway*

Innervation of the rat soleus muscle changes in the first two weeks after birth from a ... where each muscle fiber is innervated by an average of five separate nerve fibers (polyneuronal innervation) to the adult state in which each muscle fiber is singly innervated (Redfern 1970). Although this elimination of innervation is known to occur because a constant number of motor neurons each reduce the number of muscle fibers they innervate (Brown *et al.* 1976), little is understood about its cause.

By blocking the conduction of nerve impulses in the sciatic nerve we have investigated whether the functional activity of the synapses in neonatal muscles is required for the elimination of polyneuronal innervation. Nerve blocks were accomplished by insertion into the sciatic nerve of a plastic pellet (Sanger et al. 1976) containing tetrodotoxin (TTX), an agent which blocks nerve impulses. Using these TTX pellets, we have obtained total block of impulses in the soleus motor axons for up to 9 days.

We have found that such blocks initiated in 9 or 10-day old animals (just prior to the appearance of the first singly innervated ...

of oocyte maturation 2) that other LH-induced changes in the granulosa cells are responsible for oocyte maturation and 3) that LH induced increases in granulosa cell cyclic AMP levels thus *indirectly are of importance for resumption of oocyte maturation.*

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C 25

Colour Sensitivity of Fly s Retina

By M JÄRVILEHTO J MORING and KRISTINA MORING *Department of Physiology University of Oulu Finland*

Behavioral studies have shown that many insects possess a colour vision (v Frisch 1965) In the fly's retina at least two visual pigments with different absorption maxima have been found by using microspectrophotometry Electrophysiological methods revealed three major groups of receptor cells with different sensitivity maxima all having a second maximum in the UV region Besides those double-peaked sensitivities cells with single-peaked sensitivity in UV have been reported by recent investigators Based on microspectrophotometry and genetical selection experiments with *Drosophila* it has been concluded

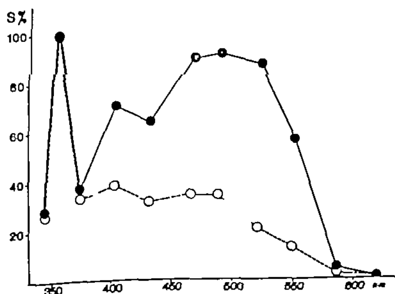


Fig. 1 The relative spectral sensitivity of second order neurons. ● UV VIS cells ○ UV cells.

that all R1–6 cells could have double-peaked spectral sensitivities and only the R7 single peaked UV sensitivity

In this study we measured the spectral sensitivity of individual receptors and second order neurons in retina of fly *Calliphora*. Intracellular recordings were carried out with glass capillary microelectrodes filled with Procion Yellow solution. In total 23 receptors and 25 second order neurons were recorded and identified. Three groups of receptors were found: cells with high UV (353 nm) and very low visible region sensitivity (1 0.03) cells with high UV and low visible region sensitivity (1 0.85) and cells with low UV but high visible region sensitivity (0.65 1). Two major groups of second order neurons were found: cells with high UV and relatively low visible region sensitivity (1 0.35) and cells with high sensitivity in both regions (1 0.94). The sensitivity recordings were made in non-saturated region of the intensity-response function. The spectral sensitivity of the cell was not found to be attributed to the same morphological structure in different preparations. These preliminary results give a rise for assumption that a revision of morphological classification is necessary according to the cell function.

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C 26

The Influence of Nerve-muscle Activity on the Elimination of Synapses in Neonatal Rat Soleus Muscle

By W. THOMPSON D KUFFLER and J K. S. JANSEN *From the Institute of Physiology University of Oslo Norway*

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We have found that such blocks initiated in 9 or 10-day old animals (just prior to the appearance of the first singly innervated fibers) do not prevent the appearance of singly

of oocyte maturation 2) that other LH-induced changes in the granulosa cells are responsible for oocyte maturation and 3) that LH-induced increases in granulosa cell cyclic AMP levels thus *indirectly* are of importance for resumption of oocyte maturation

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C 25

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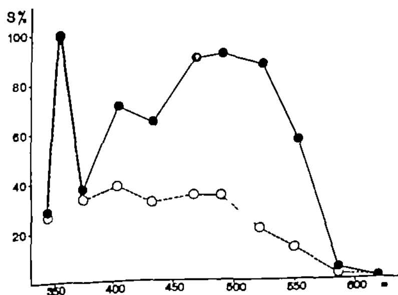


Fig. 1 The relative spectral sensitivity of second order neurons. ● UV VIS cells ○ UV cells.

were used. In both positions the depolarizing effect seemed to be more effective than the hyperpolarization in diminishing the IPSP amplitude, membrane resistance as well as the firing frequency The hyperpolarizing, but not the depolarizing effect of GABA, could be reversed by passing currents across the cell membrane. The reversal potential for GABA applied at the soma was the same as for the IPSP Addition of 10^{-4} mM bicuculline reduced both GABA response types and changed the orthodromic response from a single spike to a long burst of discharges.

The results support the idea that the hyperpolarizing basket cell inhibition in the hippocampus is mediated by release of GABA on the somata of pyramidal cells (Curtis *et al.* 1970). In addition, there may be interneurones that release GABA on dendrites, causing localized inhibition by a conductance shunt mechanism. This would fit with the accumulation of glutamate decarboxylase both near the soma and at certain dendritic positions (Fonnum and Storm-Mathisen 1969).

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C 28

Delayed Drinking Induced by Electrical Stimulation of the Medial Forebrain

By M. Rundgren *Department of Physiology I Karolinska Institutet Stockholm Sweden*

Strictly stimulus-bound drinking was previously induced by unipolar electrical stimulation within the perifornical region of the hypothalamus in the goat (Andersson and McCann 1955). However the integrated results of later studies in the same species imply that cerebral sodium sensitive receptors regulating water intake predominantly are located more medially close to the anterior border of the 3rd ventricle (*cf* Andersson 1977) Therefore, it was of interest to include this area in a field of bipolar electrical stimulation in the conscious goat.

Two goats were used for expts over a period of 8 weeks. Each animal had a pair of stainless steel electrodes permanently implanted bilaterally into the medial forebrain (3 mm apart and with 3 mm uninsulated tips) at the level of the anterior commissure Modified Hess technique was employed with stimulus parameters: 0 4–3 mA, 50 c/s pulse duration 1.5 ms stimulation periods 1 10 min.

One goat invariably responded with drinking to current strength of 1 mA or higher Drinking generally started 20–30 s after stimulation, and 0.5 to 2.5 liters of water were drunk. The water consumption was roughly proportional to the amount of current applied. When the animal was not allowed to take significant amounts of water after the stimulations obvious urge to drink remained for 8 to 10 min. Stimulations up to the strength of

[illegible]

innervated fibers in the muscle although the rate of their appearance is reduced. On the other hand 5–6 days after the initiation of nerve block a reversal of synapse elimination was noted with the reappearance of more polyneuronally innervated fibers.

We conclude that the functioning of the synapses (or the muscle activity associated with it) is not required for elimination to occur Extended nerve block, however is able to reverse elimination and produce nerve sprouting.

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C 27

Two Separate Effects of GABA on Hippocampal Pyramidal Cells *in vitro*

By I A LANGMOEN P ANDERSEN L. GJERSTAD A. MOSFELDT LAURSEN and T GANES *Institute of Neurophysiology University of Oslo*

The aim of the present study was to examine the mode of action of gamma-amino butyric acid (GABA) on hippocampal pyramidal cells. Intracellular recordings were taken from CA1 pyramids in hippocampal slices *in vitro* (Skrede and Westgaard 1971) Two different effects of iontophoretically applied GABA were observed when applied close to the soma, GABA hyperpolarized the cell whereas it depolarized the neurone when delivered in the dendritic tree In both cases were the potential changes associated with an increase of membrane conductance reduced IPSP amplitude and cessation of spontaneous and evoked discharges. (Fig 1)

The depolarization which followed ejection of GABA among the dendrites changed to a hyperpolarization lasting for the duration of the ejection. The hyperpolarization following soma ejection was followed by a depolarization when stronger GABA ejection currents

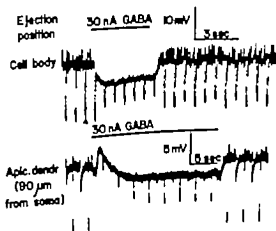


Fig. 1 Ejection of GABA at the cell body (upper half) and in the apical dendritic layer (lower part). Both effects were accompanied with a fall in membrane resistance (▲). 30 nA ejecting current 15 nA backing current. IPSP (●).

DEMONSTRATIONS

D 1

RV/TLC Ratios Measured with ^{133}Xe-radiospirometry and He-dilution Methods

By K. KAUPPINEN-WALIN, H. HOLLI, A. R. A. SOVIJÄRVI and A. UUSITALO. *Department of Biomedical Sciences, University of Tampere, and Department of Clinical Physiology, Tampere Central Hospital, Tampere, Finland*

^{133}Xe-radiospirometry has been shown to be a clinically suitable method for acquiring information on the distribution of ventilation, the perfusion and the ventilation/perfusion ratio of the lungs. The validity of a multidetector type radiospirometer for acquiring quantitative data of lung volumes has, however, been discussed. But regardless of whether the volumes *per se* are valid, their ratios should be no different from the ratios conventionally determined. And the residual quota is quite useful in evaluating the degree of a patient's hyperinflation.

To establish the reliability of radiospirometrically determined residual quotas we measured the RV/TLC of 30 healthy people with a multidetector type ^{133}Xe-radiospirometer on one hand and with a He-dilution in closed circuit on the other hand. The methods were similar in that they both employ a foreign gas for an indicator to calculate the volumes RV and TLC.

The subjects comprised 13 young people, 19 to 1 years old, seven women and six men and 17 old people, 65 to 70 years old, nine women and eight men. The RV/TLC ratio was determined for each subject with both methods, both in sitting and in supine positions.

The radiospirometric results on RV/TLC determinations showed no differences between individual first and second determinations. Neither were any significant differences between sitting and supine RV/TLC *ratios exposed. Thus, we analyzed the data obtained with the first measurement* by both methods as done in clinical routine, with the He-dilution method it was the first in the sitting position and with radiospirometry the first in the supine position.

Statistical analysis (paired observation t-test) showed that the residual quota determined with the two method, He-dilution and ^{133}Xe-radiospirometry, was essentially the same (P> 0.1).

Thus it seems that RV/TLC ratios produced automatically along with routine radiospirometric lung function examinations with the multidetector type ^{133}Xe-radiospirometer are valid data for estimation of the degree of a patient's possible hyperinflation.

2 mA did not induce any other obvious behavioural effects On repeated stimulations within two hrs there was a gradual attenuation of the drinking response Likewise pre stimulatory hydration of the goat markedly reduced its sensitivity to stimulation. A similar but less pronounced and consistent dipsogenic effect was obtained in the second goat. *Here drinking commenced 1 to 2 min after discontinuation of the electrical stimulation.*

The delayed drinking induced by electrical stimulation in the present experiments is different from the strictly stimulus-bound drinking earlier obtained in response to unilateral hypothalamic stimulation (Andersson and McCann 1955) An explanation of the difference might be that the previous unilateral stimulations mainly affected immediately responding efferent pathways from "thirst-receptors" located close to the ventricular wall and that these receptors for some reason react with post-stimulatory excitation to the application of electrical impulses The present field stimulation may gradually have induced a local redistribution of Na sufficient to activate the thirst mechanism in the post stimulatory period

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no less important in work on smooth muscle than on striated muscle The apparatus scribed here is used with smooth muscle strips of 1–4 mg weight and 3–7 mm length veloping forces of 6–30 mN Change from isometric to isotonic conditions can be ac mplished in less than 2 ms and length oscillations after the step usually do not extend er more than 10 ms, for small force steps considerably less A 20 mm long lever made m stainless steel wire, is mounted on micro ball bearings It is tapered from a diameter 0.7 mm near the fulcrum to 0.2 mm near the tip Afterload is provided by an adjustable ne spring acting on the lever close to the fulcrum. A photoelectric displacement trans- cer (sensitivity 10 V/mm) is influenced by a small vane mounted on the lever axis. The uivalent mass of the moving parts is 8 mg. The lever is hanging vertically with its tip mersed in the muscle bath. Two independently adjustable stops acting in the forward d backward directions, make contact with the lever in the solution 1 4 mm above the int of muscle attachment, thereby eliminating adhesion. The stops can be released by ectromagnetic relays, and are checked by micrometers permitting either free isotonic leases or controlled length steps. Due to its small dimensions the lever will bend some- hat hen an afterload force (up to 30 mN) is applied causing a shift in baseline of the splacement transducer A force of the same magnitude applied at the muscle attachment ll, however cause only negligible deformation (0.2 μm/mN) due to the position of the ops Therefore withdrawal of the stops as in a quick release will not appreciably alter e strain on the lever The muscle is mounted horizontally with its ends wrapped into mall pieces of aluminum foil, one of which is hooked on to the sharpened tip of the lever d the other to a similar tip connected to a capacitance force transducer (sensitivity 0 1 /mN compliance 0.2 μm/mN natural frequency 620 Hz) The bath is a thermoregulated d perspex trough perfused with oxygenated solution Contraction of the muscle is in- ced by means of electrical stimulation, K -high solution or drugs. The whole mechani- l arrangement is rigidly mounted on a heavy iron plate

D 4

atecholamine Content in Blood Plasma, Heart and Intercostal Arteries of the African Lung- sh

By T ABRAHAMSSON S. HOLMGREN S NILSSON and K. PETTERSSON *Department of Zoophysiology University of Göteborg S eden*

he chromaffin tissue which corresponds to the catecholamine secreting adrenal medulla f the mammals, shows a variable arrangement among the lower vertebrates. In the cyclo- omes chromaffin cells are found in large quantities within the heart and also in the lls of the cardinal veins. The elasmobranches ha e chromaffin cell groups arranged seg- entally in connection with the sympathetic ganglia and the chromaffin tissue in the eosts lies mainly within the anterior part of the kidney (head kidney) often lining the ls of the posterior cardinal veins.

The chromaffin tissue of the African lungfish, *Protopterus* first demonstrated by Gia- ma (1906) and Holmes (1950) is located in the walls of the intercostal branches of the

D 2

Tissue Osmolality in the Papillae of the Cat s Tongue

By DAN AXEL HALLBACK MATS JODAL and OVE LUNDGREN *Department of Physiology University of Göteborg Sweden*

The tongue papillae have a vascular arrangement which resembles that of the intestinal villi *i e* they are supplied by an arterial vessel that runs centrally to the tip where it branches into a dense capillary network. The two blood flows running in close approximation and in opposite direction to each other constitutes the prerequisite for the presence of a countercurrent exchanger in the papillae. Such a mechanism may act by concentrating absorbed food substances at the papillary tips and may thereby be of importance for the sensation of taste

To test this hypothesis the tissue osmolality at different levels of the papillary core was determined with a cryoscopic method described by Jodal *et al* (1977) The surface of cat s tongue was flushed for at least 30 min with isotonic Krebs solutions containing different amounts of sodium and glucose The tongues were then momentarily frozen in isopentane precooled in liquid nitrogen and cut obliquely in 15 μm thick sections so as to give cross-sections at different levels of the papillae By stepwise increasing the tissue temperature the tissue osmolality could be determined as a freezing point depression in the papillary core

Both filiform and fungiform papillae showed a marked osmolar gradient from tip to base of the papillae when flushed with an isotonic Krebs glucose solution the tip values being about 600–800 mOsm/kg H_2O Two different types of filiform papillae were studied in more detail In one type being large and horny a significant decrease in tip osmolality occurred when glucose was replaced by mannitol Exchanging also the sodium ions with choline reduced the tip osmolality to the same value as found in *in vitro* condition *i e* about 400 mOsm/kg H_2O In the other type of filiform papillae no change in the osmotic gradient was seen with the replacement of either sodium and/or glucose The findings support the hypothesis of a presence of a countercurrent exchanger in the tongue papillae and they also provide evidence for different electrolyte transport mechanisms in different papillae

Reference

JODAL, M D A. HALLBACK and O LUNDGREN Tissue osmolality in intestinal villi during luminal perfusion with isotonic electrolyte solutions *Acta Physiol Scand* In press

D 3

An Apparatus for Mechanical Experiments on Isolated Smooth Muscle

By L. SJÖLIN P HELLSTRAND and B CLEMENTZ. *Department of Physiology and Biophysics University of Lund Sweden*

Isotonic quick release and quick stretch experiments on muscle require an experimental design to minimize inertial forces if disturbing length oscillations are to be avoided This

is no less important in work on smooth muscle than on striated muscle The apparatus described here is used with smooth muscle strips of 1–4 mg weight and 3–7 mm length developing forces of 6–30 mN Change from isometric to isotonic conditions can be accomplished in less than ms and length oscillations after the step usually do not extend over more than 10 ms, for small force steps considerably less. A 20 mm long lever made from stainless steel wire is mounted on micro ball bearings. It is tapered from a diameter of 0.7 mm near the fulcrum to 0.2 mm near the tip Afterload is provided by an adjustable plate spring acting on the lever close to the fulcrum. A photoelectric displacement transducer (sensitivity 10 V/mm) is influenced by a small vane mounted on the lever axis. The equivalent mass of the moving parts is 8 mg. The lever is hanging vertically with its tip immersed in the muscle bath. Two independently adjustable stops, acting in the forward and backward directions make contact with the lever in the solution 1 4 mm above the point of muscle attachment, thereby eliminating adhesion. The stops can be released by electromagnetic relays and are checked by micrometers permitting either free isotonic releases or controlled length steps. Due to its small dimensions the lever will bend somewhat when an afterload force (up to 30 mN) is applied causing a shift in baseline of the displacement transducer A force of the same magnitude applied at the muscle attachment will, however cause only negligible deformation (0.. μm/mN) due to the position of the stops. Therefore, withdrawal of the stops as in a quick release will not appreciably alter the strain on the lever The muscle is mounted horizontally with its ends wrapped into small pieces of aluminium foil, one of which is hooked on to the sharpened tip of the lever and the other to a similar tip connected to a capacitance force transducer (sensitivity 0.1 V/mN compliance 0.2 μm/mN natural frequency 620 Hz) The bath is a thermoregulated 2 ml perspex trough perfused with oxygenated solution. Contraction of the muscle is induced by means of electrical stimulation, K -high solution or drugs The whole mechanical arrangement is rigidly mounted on a heavy iron plate

D 4

Catecholamine Content in Blood Plasma Heart and Intercostal Arteries of the African Lungfish

By T Abrahamsson S Holmgren S Nilsson and K. Pettersson *Department of Zoophysiology University of Göteborg S eden*

The chromaffin tissue, which corresponds to the catecholamine secreting adrenal medulla of the mammals, shows a variable arrangement among the lower ertebrates In the cyclostomes, chromaffin cells are found in large quantities within the heart, and also in the walls of the cardinal veins. The elasmobranches have chromaffin cell groups arranged segmentally in connection with the sympathetic ganglia, and the chromaffin tissue in the teleosts lies mainly within the anterior part of the kidney (head kidney) often lining the walls of the posterior cardinal veins.

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Both filiform and fungiform papillae showed a marked osmolar gradient from tip to base of the papillae when flushed with an isotonic Krebs-glucose solution the tip values being about 600–800 mOsm/kg H_2O Two different types of filiform papillae were studied in more detail In one type being large and horny a significant decrease in tip osmolality occurred when glucose was replaced by mannitol Exchanging also the sodium ions with choline reduced the tip osmolality to the same value as found in *in vitro* condition *i.e* about 400 mOsm/kg H_2O In the other type of filiform papillae no change in the osmotic gradient was seen with the replacement of either sodium and/or glucose. The findings support the hypothesis of a presence of a countercurrent exchanger in the tongue papillae and they also provide evidence for different electrolyte transport mechanisms in different papillae

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D 3

An Apparatus for Mechanical Experiments on Isolated Smooth Muscle

By L. SJÖLIN P HELLSTRAND and B CLEMENTZ. *Department of Physiology and Biophysics University of Lund Sweden*

Isotonic quick release and quick stretch experiments on muscle require an experimental design to minimize inertial forces if disturbing length oscillations are to be avoided This

& BD). The present study reports upon the nature of the receptors responsible for these fects.

Rabbits were anesthetized with chloralose and urethan and placed on their back with e head fixed to the table. Changes in position of the left maxillary incisor were measured th ultrasonic technique as before

Responses of the tooth to bilateral stimulation of the cut cervical sympathetic nerves V 1 ms, 2–20 Hz) were reduced by i.v. phenoxybenzamine (above 3 mg/kg) largely olished by phentolamine (2–3 mg/kg). Remaining responses disappeared after infusion propranolol (0.3–1.5 mg/kg). When not preceded by phenoxybenzamine propranolol creased the responses to nerve stimulation. Atropine was without effect.

Propranolol induced a rapid extrusion, of a magnitude greatly exceeding that resulting om mechanically evoked rises in arterial or venous pressure (aortic balloon inflation, clusion of jugular veins). Isoprenaline (0.3–0.6 mg/kg) reversed or prevented this movement.

These results can be explained by the existence of a double adrenergic control of vessels regulating the axial position of the tooth, sympathetically innervated alpha-receptors effecting constriction of pre-capillary vessels and beta-receptors effecting dilatation of ost-capillary resistance vessels. The latter type of receptors must be activated mainly y humoral agents. Changes in position of the tooth presumably reflected alterations in apillary blood volume or tissue pressure. The recently demonstrated importance of pressure and tone in periodontal vessels for the position of the rabbit's incisor (Myhre, L., L. R. Preus and H. Aars, *Acta physiol. scand.* 1977, this meeting) suggests that the presently observed receptors were located to the periodontal rather than the periapical or pulpal, vascular bed.

D 6

Mechanisms Controlling Mobility and Position of the Rabbit's Tooth

By L. Myhre, H. R. Preus and H. Aars. *Department of Physiology and Biochemistry, Dental Faculty, University of Oslo, Oslo, Norway*

Teeth are known to be firmly "kept in place"—yet they move when subjected to loads exceeding a few grams. This dynamic resistive property of the tooth has been ascribed to the collagenous fibres and the vasculature of the periodontium, the pulpal tissue pressure, or to the periapical tissue. We have investigated the mobility of the rabbit's incisor and the localization of the tissue determining this mobility by recording the movements of the incisor in response to axial loads before and after the tooth had been cut close to the apex. We also studied the movements induced by vasoactive drugs and changes in systemic arterial blood pressure.

The rabbits were anesthetized with chloralose and urethan, and placed on their back with the head fixed to the table. Positional changes of the left maxillary incisor were recorded by ultrasound transit time technique: one crystal was fastened to the tooth, the other to the skull by a metal bridge. Loads of 5–20 g and 20 s duration were applied by

dorsal aorta. A sympathetic innervation of this tissue was also described by Holmes (1950)

As part of a comparative study of adrenergic mechanisms in lower vertebrates conducted in this laboratory a preliminary study has been carried out in a few specimens of the African lungfish *Protopterus aethiopicus*

Samples of blood plasma, the heart and the intercostal arteries were assayed spectrofluorimetrically for adrenaline and noradrenaline. The concentrations of adrenaline and noradrenaline were very similar in the plasma of control animals (2.3±0.8 and 2.4±1 1 μg/100 ml plasma respectively means ±S E. n=6) but after "stress" induced by chasing the animal around its tank for about 15 min the noradrenaline concentration was increased more than 20-fold while the adrenaline level was increased only 4-fold (means of two animals only)

Large quantities of adrenaline and especially noradrenaline (4.2±1 1 and 70.8±17.3 μg/g tissue respectively· means ±S E. n=6) were found in the heart. These high levels compare with the situation in cyclostomes (Augustinsson *et al* 1956 Euler and Fänge 1961) Much lower quantities of the catecholamines were found in the intercostal arteries in this initial study (adrenaline 0.3±0 1 μg/g, noradrenaline <0 1 μg/g tissue means ±S.E. n=8)

In conclusion the preliminary data so far obtained indicate a storage of large quantities of especially noradrenaline in the heart, a situation resembling that in cyclostomes. We have been unable with the present method to find but smaller quantities of catecholamines in the intercostal arteries where chromaffin tissue has been described by Giacomini (1906) and Holmes (1950)

Further extensive work is needed to clarify the correlation between the high noradrenaline levels in the heart and in the plasma after "stress" and the nature of the previously described chromaffin tissue in the intercostal arteries

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D 5

Adrenergic Receptors in Periodontal Vessels

By H AARS. *Department of Physiology and Biochemistry Dental Faculty University of Oslo Oslo Norway*

The position of the rabbit's incisor is tonically controlled by sympathetic nerves cutting the cervical sympathetic nerves makes the tooth move out of its socket (extrusion) while stimulation of the nerves causes intrusion (Aars H *Acta physiol scand* 1976. Suppl.

oocytes that had started to mature showed an increased oxygen consumption as compared to the dictyate oocytes. Following culture in the absence of hormone the cumuli showed a transient decrease in oxygen consumption at 1 hr of culture; when LH was present in the medium this decrease was continuous. Oocytes resumed meiosis "spontaneously" after 1 to 2 hrs in culture. At the same time an increase in oxygen consumption was recorded.

The present study shows that there is a close correlation in time between the metabolic changes in the oocyte and in the cumulus cells both following *in vivo* and *in vitro* stimulation, indicating that changes in energy metabolism in these two follicular cell types might be of importance for the resumption of meiosis. The lag period observed following stimulation with LH *in vivo* as compared to the rapid response during *in vitro* culture might reflect that LH *in vivo* has to counteract an inhibitory influence which is rapidly removed by isolation but needs longer time for inactivation *in vivo*.

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D 8

Differences between Near-threshold Membrane Currents in Slowly and Fast Adapting Lobster Stretch Receptor Neurones

By W. Grampp. *Department of Physiology and Biophysics, University of Lund, Sweden*

Near-threshold membrane currents were determined in slowly and fast adapting lobster stretch receptor neurones in an attempt to explain these cells' differing firing characteristics. In this connexion major dissimilarities in the presence and control of these currents were found in the two cell types. Some of these dissimilarities are illustrated in Fig. 1 showing the steady state voltage dependence of threshold and subthreshold membrane currents in the slowly and fast adapting cell.

Further differences were related to the facts (1) that a transiently activateable 4-aminopyridine sensitive K current was in near-threshold potential regions present mainly in the fast adapting cell and (2) that a fraction of the TTX sensitive Na current associable with the generation of membrane noise (Sjölin and Grampp 1975) was observeable only in the slowly adapting cell.

In steady state the voltage dependence of the various membrane currents proved to be of such a kind that in the slowly adapting cell it gave rise to the formation of a negative slope of the relationship between membrane polarisation and total ionic membrane current in suprathreshold potential regions. In this way conditions were created making possible

means of a rod connected to an electromagnet Blood pressure was measured with a catheter in the brachial artery

The amplitude of inward movements (intrusion) increased with increasing loads Intrusion and extrusion after release of the load showed an initial rapid phase followed by a slower less pronounced further movement Neither amplitude nor pattern of movements were altered after sectioning of the tooth or during the first two days after the animal had been killed We therefore conclude that the mobility of this continuously erupting tooth was determined by the properties of the periodontal collagenous fibres.

A rise of blood pressure produced by inflation of an intra-aortic balloon resulted in *extrusion of the tooth* Norepinephrine i v raised arterial pressure but led to intrusion of the tooth whereas i v papaverine caused a slight extrusion if pressure was maintained by means of the balloon Since the pressure-induced extrusion remained unchanged after the tooth had been cut but was greatly diminished by infiltration of the periodontium with norepinephrine the responses of the tooth to alterations in pressure and vascular tone most likely were due to changes in vascular volume or tissue pressure in the periodontium.

D 7

Further Studies on the Metabolism of the Preovulatory Rat Oocyte and Cumulus Oophorus

By C MAGNUSSON and T HILLENSJÖ *Department of Physiology University of Göteborg Sweden*

The preovulatory surge of LH induces the resumption of oocyte meiosis (oocyte maturation) as well as morphological changes in the cumulus cells The meiotic process proceeds from the dictyate stage up to metaphase II close to ovulation The mechanism for the LH effect on oocyte maturation is not yet clarified and neither is the primary target cell for this action localized It has earlier been shown that LH acutely stimulates aerobic glycolysis of the whole preovulatory follicle (Nilsson 1974) and decreases the oxygen consumption of the cumulus cells (Dekel *et al* 1976) In a recent study it was furthermore shown that the oxygen consumption of the oocyte itself increases following the onset of oocyte maturation (Magnusson *et al* 1977)

The aim of this study was to analyze more closely the temporal relationship between the respiratory changes *in the oocyte itself and in the cumulus cells Oocytes and cumuli* were obtained from immature rats pre treated with a low dose of pregnant mare s serum gonadotrophin two days earlier The isolated cells were placed in a minute gas-tight chamber containing medium with hemoglobin as an indicator of oxygen tension and the oxygen consumption by the cells recorded according to Hultborn (1974) Oocyte maturation was induced *in vivo* by i p injection of 10 μg NIH LH-S18 Oocyte maturation was also induced artificially by isolating the oocytes from the follicles into culture ("spontaneous maturation")

Following the LH injection oxygen consumption of the isolated cumulus remained unchanged for 3 hrs Four hours after the injection a significant decrease was seen In the oocytes the first significant change occurred 3 to 4 hrs after the LH injection At this time

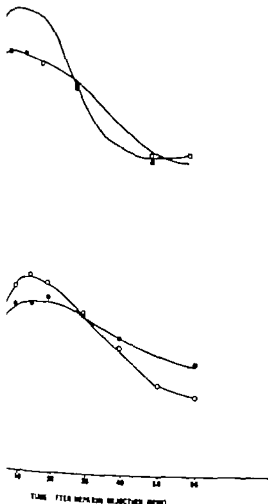


Fig. 1 Total lipolytic activity of postheparin plasma in two test subjects. Subject A (circles): male, age 29 years height 186 cm and weight 74 kg; B (squares): male, 34 years, 166 cm, 104 kg. Open symbols represent the activity at rest and the closed ones during physical exercise.

familial hypertriglyceridemias but the role of physical exercise as a possible ef
f triglyceride lipases has deserved less attention.
e present study the enzymatic activity was determined using ^{14}C-triolein as sub-
s described by Huttunen *et al* (1975) with slight modifications. For the selective
ement of hepatic and extra-hepatic (lipoprotein) lipase the inhibition of lipoprotein
y 1.0 mol/l NaCl was utilized No specific antiserum was used. In the experiments
c cannula was inserted into an antecubital vein of the subjects. They pedaled an
ally braked bicycle ergometer against light load for 5 min. After heparin injection
/kg) the load was increased to represent about 45 % of the subject's maximal oxygen

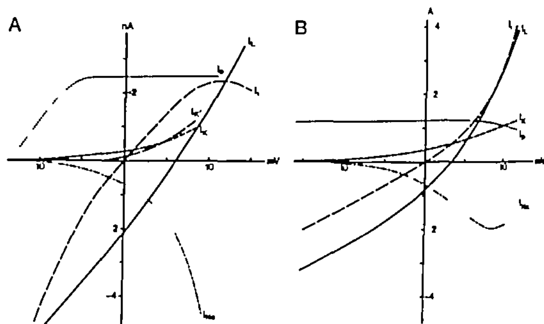


Fig 1 Steady state voltage dependence of membrane currents in the slowly (A) and fast (B) adapting lobster stretch receptor neurone I_t, total ionic current; I_{Na} TTX sensitive Na current I TEA sensitive K current; I Co sensitive TEA resistant K current I_L, leak current and I_p, pump current. Resting potential (corresponding to origo) at −65 mV in A and −67 mV in B

the generation of maintained repetitive impulse activity in response to long-lasting suprathreshold stimuli In the fast adapting cell the slope of the steady state relationship between membrane polarisation and total ionic membrane current was found to be positive at all potentials This may explain the cell's inability to maintain repetitive impulse firing during prolonged suprathreshold stimuli

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D 9

Triglyceride Lipase Activities of Human Postheparin Plasma in Obese and Non-obese Subjects in Physical Exercise

By J MARNIEMI P PELTONEN I VUORI and E HIETANEN *The Rehabilitation Examinations Centre of the Social Insurance Institution 20720 Turku 72 and Department of Physiology University of Kuopio 70100 Kuopio 10 Finland*

Heparin injection is known to release lipolytic enzymes into human circulation (Korn 1959) These enzymes originate partly from the capillary endothelium and partly from the liver (Huttunen *et al* 1975). The enzymes have been studied especially in obesity and in

from the three stains was then compared. Based on the NADH D stain 67% (47–87) of the fibres were classified as FTa and FTb in agreement with the ATPase (pH=4.6) stain. The corresponding result from the αGP D stain was 41% (11–81). Only 28% (11–59) were classified identically in all three histochemical stainings. The most inconsistent results were obtained from the orienteers where the number of FTb fibres was over estimated from the NADH D and αGP-D stains.

In conclusion: human skeletal muscle is composed of 2 major fibre types, but subgroups definitely do exist. When using histochemical stainings for classification of FTa and FTb fibres it is, however, difficult to ascertain any other way than using an ATPase (pH=4.6) stain.

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D 11

Effect of Hypoxia in Vivo on the Concentrations of Certain Metabolites in Rat Liver Cytosol and Mitochondria

By V. L. Kinnula and I. Hassinen. *Departments of Physiology and Medical Biochemistry, University of Oulu, Finland*

The tissue concentrations of certain metabolites were measured after varying periods of hypoxia in order to obtain a better understanding of the reactions of the energy metabolism under such conditions. When the oxygen supply is inadequate the cellular redox state can be expected to become more reduced. The indirect metabolite technique enabled the free NADH/NAD ratio to be calculated from the ratio of the measured concentrations of the oxidized and reduced substrates of a compartmentalized NAD-linked dehydrogenase, and the redox states of the cytosol and mitochondria could be determined separately by using appropriate redox state couples.

Adult Sprague-Dawley rats were anesthetized with pentobarbital (60 mg/kg, intraperitoneally) and after cannulation of the trachea the respiration was controlled using a rodent respiration pump. The animals breathed room air for 30 min in the control experiments and 10–11% O_2 in nitrogen in the hypoxia experiments. Some of the hypoxia groups had been placed in a hypobaric pressure chamber at 50.5 kPa for 24 h prior to the 30-min period in the respirator. At the end of this controlled ventilation in the respirator one lobe of the liver was freeze-clamped, and the following metabolite ratios were determined enzymatically: lactate/pyruvate, α-glycerophosphate/dioxyacetonephosphate and 3-hydroxybutyrate/acetoacetate.

All the redox pairs measured became more reduced under hypoxic conditions. After the acute 30-min period in the respirator under 10–11% O_2 the lactate/pyruvate ratio increased from the control value of 13.5 to a figure of 80.4, the α-glycerophosphate/dioxy-

uptake previously determined The heart rate of the subjects was 110–130/min, and the duration of the exercise was 60 min

The reproducibility of the time activity curves of the total lipolytic activity and of the hepatic and extra hepatic lipases after heparin administration was good both at rest and during exercise However rather marked inter individual differences were observed both in the form of the curves and in the peak activity

The physical exercise used appeared not to exert any clear-cut changes on the enzyme activity curves However in obese subjects the peak activities were 40–60% higher than in non-obese subjects and the relative increase was most remarkable in extra-hepatic lipoprotein lipase Typical enzyme activity curves are shown in Fig. 1

According to the present results the immediate response to physical exercise of the lipolytic activity released by heparin is small Studies concerning possible adaptive effects of long-term physical training on enzymes of triglyceride metabolism are now in our research program

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D 10

Human Skeletal Muscle: Subgrouping of Fast Twitch Fibres

By G SJØGAARD M HOUSTON E. NYGAARD-JENSEN and B SALTIN *August Krogh Institute University of Copenhagen Copenhagen Denmark*

It is generally accepted that human skeletal muscle is composed of two major fibre types slow twitch (ST) and fast twitch (FT) An ongoing discussion is. what speaks in favor of 2 subgroups of human FT fibres in accordance with other species

From histochemical stainings for myofibrillar ATPase (Brooke and Kaiser 1970) FT fibres can with great certainty be classified into two groups Based on this classification significant differences of FTa and FTb fibres have been demonstrated with respect to 1) cross sectional area, 2) recruitement pattern and 3) biochemical analyzed enzyme activities (Essén *et al* 1975)

In the present study it was investigated whether the subgroups of FT fibres can be identified reliably based on histochemical stainings for oxidative and glycolytic enzyme activities. Muscle biopsy samples were obtained from the quadriceps femoris muscle (vastus lateralis) from 4 untrained women 4 trained men and 4 well trained orienteers. Serial cross sections were histochemically stained for NADH D αGP-D and myofibrillar ATPase (after preincubation of respectively pH = 10.3 4 6 and 4.3) FT fibres were identified from the ATPase (pH = 10.3 and 4.3) stains and then divided into two groups based on the staining intensity of NADH D αGP D and ATPase (pH = 4 6) Three persons worked independently each with one of the three stains The classification of each fibre

D 13

Rapid Determination of Cardiac Output in Small Animals from Dye Dilution Measurements

By L. STAGE. *Department of Physiology University of Göteborg Sweden*

Everybody who has used the dye dilution method for cardiac output (CO) determination knows that the calculations are quite time consuming, or involve expensive instruments. An inexpensive system, primarily designed for the use in rats, is described which makes it possible to perform frequent CO determinations with calculation of the CO values within one minute after the injection, strictly according to the Stewart Hamilton method. Ordinarily the most time consuming moments in this method are the logarithmic plotting and replotting of the density curve together with the determination of area under curve. However if it is known where the density curve is monoexponential, the area under the curve can be rapidly determined with an integrator and two points on the density curve.

The system for CO measurement consists of a step dispenser injection device, a densitometer with a suction arrangement, an electronic unit, a three channel writer and a programable pocket calculator. The electronic unit provides the three channel writer with signals for 1) the cardiogreen density curve 2) the integral of the curve and 3) its logarithm. The unit also includes an automatic baseline reset. From the downslope of the logarithmic curve where it forms a straight line, it can be judged which part of the density curve that is strictly monoexponential. Two points on this monoexponential part of the density curve completely determine the tail function, from which the tail area can be calculated. Now the total area under the curve is determined as the integrator's value where the tail starts plus the tail area. Calculation of flow from a few parameters and curve points involves some mathematics which are rapidly performed on a programable pocket calculator.

Two factors must be known prior to experiment, namely the sensitivity of the integrator and that of the densitometer. The sensitivity of the integrator is determined as the ratio between the integrator's deflection and the corresponding density curve area. Calibration of the densitometer can be difficult because of erythrocyte sedimentation if blood becomes stagnant in the densitometer chamber. Therefore plasma is used for calibration instead of whole blood, and the densitometer lamp is then dimmed so the same amount of light falls on the photocell as with blood. The results agree closely with the earlier used computer based way of calculating CO.

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D 14

Volumes of Saliva per Shock when Stimulating the Parasympathetic Nerve of the Dog's Submaxillary Gland at Different Frequencies

By P. GJÖRSTRUP and N. EMMELIN. *Department of Physiology and Biophysics University of Lund Sweden*

In 8 dogs under chloralose-urethane anaesthesia a submaxillary duct was cannulated and connected to a bottle in which saliva displaced distilled water. The chorda-lingual nerve

acetonephosphate ratio from 12 8 to 71 8 and the 3-hydroxybutyrate/acetoacetate ratio from 1 40 to 3 73 After the 24-h hypoxic period at 50.5 kPa followed by 30 min in the respirator under 10–11% O_2 however the corresponding ratios were markedly lower than those obtained in the animals not previously exposed to hypoxia.

The results demonstrate a significant increase in both mitochondrial and cytosolic free NADH/NAD ratios even in moderate hypoxia, but these redox changes are smaller in partially adapted animals

D 12

Hepatic Lipogenesis in the Rat under Chronic Severe Hypoxia

By V L. Kinnula M J Savolainen and I Hassinen *Departments of Physiology and Medical Biochemistry University of Oulu Finland*

Severe hypoxia has been observed to cause triglyceride accumulation in rat liver and one reason for steatosis seems to be a decrease in fatty acid oxidation capacity (Kinnula and Hassinen 1977) Another possible reason for this fat accumulation is increased lipogenesis in the liver

In this study we measured hepatic lipogenesis *in vivo* by determining the incorporation of ^3H into long-chain fatty acids as described by Lowenstein *et al* (1975) The animals were kept in a hypobaric pressure chamber at 40.8 kPa for seven days On the eighth day they were injected intracardially with 5 mCi of ^3H_2O They were then returned to the hypobaric pressure chamber still at 40 8 kPa, and liver and blood samples were obtained 60 min after the injection under ether anesthesia. Two control groups were used normal-fed and pair-fed groups The synthesis rates for hepatic fatty acids and non-saponifiable lipids were calculated as the amounts of ^3H_2O incorporated in the lipid fractions The activity of phosphatidate phosphohydrolase (EC 3 1.3 4) which seems to be a rate-limiting step in diacylglycerol and triacylglycerol formation in liver cells (Lamb and Fallen 1973) was also determined from the supernatant fraction after a seven-day hypoxic period at 40.8 kPa (Hosaka *et al* 1975 Savolainen 1977)

It was observed that lipogenesis does not increase after a hypoxic period of seven days the synthesis of hepatic fatty acids being 3 37±1 70 μmol of ^3H_2O incorporated per h per g wet wt of liver in the control group 2.31±0 97 μmol in the pair-fed control group and 2 63±0.33 μmol in the hypoxic group None of these differences were significant. Phosphatidate phosphohydrolase activity had not increased after seven days either the activity being 97 9±21 1 nmol P per min per g wet wt of liver in the control group and 79.3±25 7 nmol P in the hypoxic group the difference being non-significant.

These preliminary results indicate that chronic oxygen deficiency has no effect on hepatic lipogenesis *in vivo*

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D 15

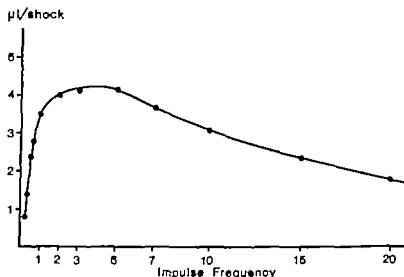
The Effect of Trauma on the Microcirculation in Skeletal Muscle

By DAVID H. LEWIS. *Clinical Research Center University Hospital Linköping Sweden*

In the presence of trauma or hemorrhage the microcirculation of normal skeletal muscle responds initially with at least three compensatory reactions mediated centrally via the sympathetic vasoconstrictor nerves. These include an increase in resistance to flow decrease in capacitance and an increase in the ratio of precapillary to postcapillary resistance. These responses are compensatory in that they help to maintain blood pressure and blood volume centrally and move extravascular fluid into the intravascular space. When skeletal muscle is itself traumatized the microcirculation of the injured muscle demonstrates an immediate, marked, transient vasodilatation which responds poorly or not at all to sympathetic vasoconstrictor stimuli and sympathomimetic agents. In anesthetized dogs (or pigs) soft tissue trauma to the hind leg causes a biphasic vasodilatory response an initial vasodilatory "spike" which after 5–15 min goes over to a more moderate vasodilatation lasting 60–90 min. The vasodilatation is not simply a release from sympathetic vasoconstrictor tone since trauma after acute post-ganglionic sympathectomy causes an additional vasodilatation. Administration of large doses of aprotinin (Trasylol®) a broad spectrum protease inhibitor before trauma reduces markedly or abolishes the "spike" response without altering the second phase. Aprotinin given after trauma has no effect. Inhibitors of serotonin, histamine (H_1-receptor) and prostaglandins given before trauma do not alter the vasodilatation. During the initial vasodilatory "spike" venous blood draining the injured area, injected close intraarterially into normal skeletal muscle, causes vasodilatation. This response can be heightened and prolonged by prior administration of dimercaprol (British antilewisite) to the recipient. Dimercaprol is known to inhibit the break down of bradykinin by inactivating carboxypeptidase. The response is not affected by giving inhibitors of serotonin, H_1-histamine receptors, or prostaglandins. The data taken together suggest that the vasodilatation seen with trauma is due to activation of the kallikrein-kinin system with release of bradykinin. Fracture of bone without accompanying soft tissue damage does not cause vasodilatation. In addition to microvascular changes there is a prolonged dilatation of the arterial tree in the injured limb extending proximal to the site of injury (ascending vasodilatation). The response to gunshot wounds is similar to that seen with soft tissue trauma. Further elucidation of the etiology and significance of the vasodilator response to trauma is of interest, since it appears not to be related to the tissues metabolic needs and may have a negative effect on the body's homeostatic mechanisms.

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was stimulated at frequencies between 0.2 and 20 Hz. Drops of water of known size falling from an outlet were recorded and the secretory rate was estimated when it had settled down to a steady level initially it was often high an effect attributed to expulsion of saliva by myoepithelial contraction (Emmelin Gjörstrup and Thesleff 1977) For each stimulation frequency the volume secreted was calculated as µl per shock The Figure shows that the largest yields per shock were obtained at stimulation frequencies between 1 and 8 Hz This is interesting in view of the fact that previous experiments on this gland indicate that maximal secretory responses to feeding are brought about by secretory impulses of frequencies which do not exceed 8 Hz at 1 Hz about 10 percent of the maximal secretory flow is obtained (Emmelin and Holmberg 1967) It is obvious that the parasympathetic acinar secretory nerves have their largest effect expressed as volume secreted per impulse within a range where they operate physiologically to evoke a fairly slow mod erate or rapid salivary flow At frequencies below 1 Hz the curve falls steeply It is possible however that here sympathetic impulses contribute causing an "augmented secretion" (Emmelin and Gjörstrup 1976)

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D 15

The Effect of Trauma on the Microcirculation in Skeletal Muscle

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In the presence of trauma or hemorrhage the microcirculation of normal skeletal muscle responds initially with at least three compensatory reactions mediated centrally via the sympathetic vasoconstrictor nerves. These include an increase in resistance to flow decrease in capacitance and an increase in the ratio of precapillary to postcapillary resistance. These responses are compensatory in that they help to maintain blood pressure move blood volume centrally and move extravascular fluid into the intravascular space. When skeletal muscle is itself traumatized the microcirculation of the injured muscle demonstrates an immediate marked transient vasodilatation which responds poorly or not at all to sympathetic vasoconstrictor stimuli and sympathomimetic agents. In anesthetized dogs (or pigs) soft tissue trauma to the hind leg causes a biphasic vasodilatory response an initial vasodilatory "spike" which after 5–15 min goes over to a more moderate vasodilatation lasting 60–90 min. The vasodilatation is not simply a release from sympathetic vasoconstrictor tone since trauma after acute post-ganglionic sympathectomy causes an additional vasodilatation. Administration of large doses of aprotinin (Trasylol®) a broad spectrum protease inhibitor before trauma reduces markedly or abolishes the "spike" response without altering the second phase. Aprotinin given after trauma has no effect. Inhibitors of serotonin, histamine (H_1-receptor) and prostaglandins given before trauma do not alter the vasodilatation. During the initial vasodilatory spike venous blood draining the injured area, injected close intraarterially into normal skeletal muscle causes vasodilatation. This response can be heightened and prolonged by prior administration of dimercaprol (British antilewisite) to the recipient. Dimercaprol is known to inhibit the breakdown of bradykinin by inactivating carboxypeptidase. The response is not affected by giving inhibitors of serotonin, H_1-histamine receptors or prostaglandins. The data taken together suggest that the vasodilatation seen with trauma is due to activation of the kallikrein-kinin system with release of bradykinin. Fracture of bone without accompanying soft tissue damage does not cause vasodilatation. In addition to microvascular changes there is a prolonged dilatation of the arterial tree in the injured limb extending proximal to the site of injury (ascending vasodilatation). The response to gunshot wounds is similar to that seen with soft tissue trauma. Further elucidation of the etiology and significance of the vasodilator response to trauma is of interest, since it appears not to be related to the tissues' metabolic needs and may have a negative effect on the body's homeostatic mechanisms.

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D 16

An Implantable Osmometer for Determination of Interstitial Fluid Colloid Osmotic Pressure

By R. K. REED and K. AUKLAND *Institute of Physiology University of Bergen Årstadveien 19 5000 Bergen Norway*

The colloid osmotic pressure of interstitial fluid (COP_i) remains an uncertain factor in the Starling equilibrium across the capillary walls To obtain a direct estimate of COP_i we have constructed an implantable colloid osmometer making use of Amicon UM10 hollow fibers As shown in Fig. 1 a 0.2 mm steel cannula provided with a sidehole is inserted into a 6–8 mm long fiber The fiber ends are glued to the cannula, which is connected to a pressure transducer through a PE 20 catheter The system is filled with saline before use

The instrument was tested against a membrane colloid osmometer (Aukland and Johnsen 1974) on 18 sera from humans and rats giving a regression coefficient of 1 04 and a correlation coefficient of 0 99

Acute implantation through a 1 6 mm (o d.) thick cannula in rats gave average pressures of −10 8 (SD 1 4 n=18) in subcutis and −8 6 mm Hg (SD 2.1 n=17) in skeletal muscle. The response time was 10–15 min both in vivo and in vitro Hydrostatic tissue fluid pressures obtained by implanting the osmometer after perforating the membrane averaged −0 65 and −0.50 mm Hg in subcutis and muscle respectively Subtracting these values gives COP_i of about 10 and 8 mm Hg in the two tissues These values are comparable to the average of 10.2 mm Hg obtained by Stromberg and Wiederhielm (1976) with chronically implanted colloid osmometers in rabbit subcutis and they agree well with COP of fluid collected by wick-technique from subcutis and muscle in rats (Johnsen 1974)

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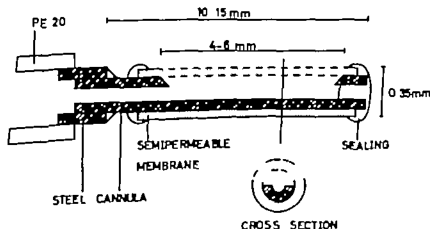


Fig. 1 Longitudinal and cross section through implantable colloid osmometer

D 17

Influence of Detergents on $Mg^{++} + Ca^{++}$-activated ATPase from Bovine Brain Microsomes

By T. SÆRMARK and H. VILHARDT *Institute of Medical Physiology C University of Copenhagen, Denmark*

In the presence of ouabain and mitochondrial ATPase inhibitors bovine brain microsomes display membrane bound ATPase activity dependent on Ca^{2+} and Mg^{2+}. It is shown that this activity can be solubilized by ionic and non-ionic detergents. Triton X 100 treatment resulting in the most stable soluble form of the enzyme.

Bovine brain microsomes were treated with deoxycholate (DOC) at various concentrations at 0°C for 20 min. at pH 8.2 and then centrifuged at 100 000×G for 60 min. at 4°C. Small concentrations (0.15%) solubilized 60% of the membrane protein but only 10% of the ATPase activity despite some activation of the enzyme. Higher concentrations were able to solubilize more of the enzyme but also inhibited the ATPase activity. At a concentration of 1% DOC 30% of the original activity could be recovered in the supernatant. The DOC-solubilized enzyme was unstable with time.

The microsomes were incubated with various concentrations of Triton X 100 at 20°C for 20 min. followed by centrifugation. Small concentrations of Triton X 100 did not activate the enzyme. At higher concentrations (0.6%) 55% of the protein of the preparation was solubilized. At the same time 50% of the initial ATPase activity could be recovered in the supernatant. The solubilized preparation was relatively stable with time. After 72 h the enzyme activity decreased to 50% of the initial activity when stored at 4°C and to 90% when stored at −22°C. On longer storage the preparation had a tendency to become cloudy however without substantial loss of activity. Addition of phospholipids to the preparation partially restored enzyme activity. Ca^{2+} (1 mM) decreased the stability of the Triton X 100 treated enzyme. Sonication of the microsomes in the presence of detergents was without effect on solubilization.

Polyacrylamide gel electrophoresis of the Triton X 100 solubilized microsomal fraction revealed 8 bands with the ATPase being the most prominent. Analytical isoelectric focusing (pH 3–10) showed 15 bands all below pH 7.5. When the preparation was subjected to preparative isoelectric focusing in granulated gel beds the ATPase activity was recovered at pH 4.8 and 6.4.

The present results show that by selecting suitable detergents and concentrations it is possible to solubilize membrane bound enzymes. This procedure opens up the possibility for further purification of the enzyme.

D 18

Rapid Restitution of Choline Acetyltransferase Activity in the Partially Denervated Urinary Bladder

By HEATHER BANNS, J. EKSTRÖM and S. P. MANN *From ARC Institute of Animal Physiology Babraham Cambridge England and Department of Physiology and Biophysics University of Lund Sweden*

D 16

An Implantable Osmometer for Determination of Interstitial Fluid Colloid Osmotic Pressure

By R. K. REED and K. AUKLAND *Institute of Physiology University of Bergen, Årstadveien 19 5000 Bergen Norway*

The colloid osmotic pressure of interstitial fluid (COP_i) remains an uncertain factor in the Starling equilibrium across the capillary walls To obtain a direct estimate of COP_i we have constructed an implantable colloid osmometer making use of Amicon UM10 hollow fibers As shown in Fig 1 a 0.2 mm steel cannula provided with a sidehole is inserted into a 6–8 mm long fiber The fiber ends are glued to the cannula, which is connected to a pressure transducer through a PE 20 catheter The system is filled with saline before use

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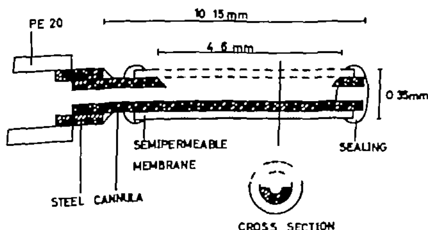


Fig. 1 Longitudinal and cross section through implantable colloid osmometer

are taken through a mask fitted around the dog's nose. The dog breathed alternating 21% 100% 21% 100% 21% O_2 for each 6 min periods. Measurements were recorded on a recorder every 2nd min for coronary blood flow (CBF) and aortic pressure (AP). From these curves mean diastolic CBF and mean diastolic AP were computed by planimetry. CBF during 21% was 31.7±5.7 (SE) ml/min and decreased significantly ($p<0.05$) to 27.8±4.5 (SE) ml/min. Conductance (CBF/AP) decreased ($p<0.05$) from .460±.078 to .384+.073 ml/min/mmHg during 21% and 100% O_2 respectively. Oxygen delivery (CBF × arterial O_2 content) remained unaffected. It is concluded that in the normal dog heart O_2 acts as a vasoconstrictor autoregulating CBF according to O_2 needs.

D 20

Leg Citrate Metabolism in Relation to Diet in Man

By E. JANSSON and L. KAIJSER. *Karolinska Institute, Departments of Clinical Physiology at Karolinska Hospital and Seraflmer Hospital, Stockholm, Sweden*

Citrate is considered as an important intermediary in the regulation of cell metabolism. If plasma free fatty acids (FFA) are increased citrate accumulates in heart and red skeletal muscle in the rat (Rennie et al. 1976). From this and in vitro experiments it is proposed that increased intracellular citrate concentration inhibits glycolysis at the phosphofructokinase step and interconversion of the inactive to the active form of pyruvate dehydrogenase (Garland et al. 1963 and Wayne et al. 1973).

The aim of present study was to relate leg citrate metabolism to type of diet. 10 healthy subjects were studied after 5 days on fat or carbohydrate rich diet. Respiratory exchange ratio, arterial-femoral venous differences of glucose, lactate, citrate and pyruvate and arterial concentrations of FFA were measured at rest and during exercise.

The respiratory exchange ratio was lower after fat diet at rest and during exercise. Arterial concentration of FFA and citrat were higher after fat and lactate and pyruvate higher after carbohydrate diet both at rest and during exercise. The release of citrate

TABLE 1. Arterial concentrations and arterial-venous differences of blood citrate and pyruvate at rest and during exercise after fat or carbohydrate diet

	Blood citrate. Fat diet	μmol/l Carb. diet	Blood pyruvate. Fat diet	μmol/l Carb. diet
artery rest	58.3±13.1	51.4±13.3*	31.9± 7.7	70.4±34**
artery exercise 5 min	58.8±12.8	53.5±13.6	136±19	164±27**
artery exercise 23 min	74.1±18.0	66.4±17.6**	143±28	181±43**
a-fv rest	−14.3± 5.8	−12.5± 4.1	+4.5± 4.8	8.4±12
a-fv exercise 5 min	−3.2± 1.5	−1.6± 1.0*	−16.7± 8.3	−6.5±10*
a-fv exercise 23 min	−3.2± 1.8	−4.0± 2.2	−5.8±12	−4.8±11

Values are mean ±SD. Levels of significant difference between fat and carbohydrate rich diet are $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***)

Recently the cholinergic innervation of the urinary bladder of the rat was studied by determining the activity of the acetylcholine-synthesizing enzyme choline acetyltransferase, after various surgical procedures (Ekström and Elmér 1977) In this connection the observation was made that the choline acetyltransferase activity was decreased somewhat in bladders examined 3 days after cutting the hypogastric nerve bilaterally but not in those examined 8 days after the operation at the later stage the enzyme activity tended in fact, to be higher in the operated bladders than in the controls The initial fall in enzyme activity was taken as evidence for the presence of cholinergic nerve fibres for the bladder in the hypogastric nerve The fact that no decrease in enzyme activity was at hand at 8 days may be explained by an increased nervous activity in remaining nervous pathways induced by the loss of the hypogastric innervation of the bladder (see Ekström 1975) The main route for the cholinergic nerves of the bladder is via the pelvic ganglion as indicated by the very profound fall in enzyme activity after bilateral extirpation of this structure The choline acetyltransferase activity has now been investigated at various times after unilateral rémoval of the pelvic ganglion to see whether also under this condition a restitution of enzyme activity from a low level could be provoked.

The enzyme activity estimated by a radioactive method was examined in bladders of adult male rats 3 6 and 25 days postoperatively At each observation the enzyme activity of 8 operated animals was compared with that of 8 control litter mates and expressed as a percentage The mean enzyme activity was (operated in % of control) at 3 days 58±4 at 6 days 75±4 and at 25 days 86±8 The percentage figures obtained at 6 and 25 days differ significantly from the figure at 3 days the p-levels being <0 02 and <0 01 respectively Thus the results show that in a bladder deprived of one of the main routes for cholinergic nerves the enzyme activity increases rapidly from a low level This increase may be restricted to the nerves present, it may also reflect collateral sprouting Both these possibilities may be attributed to enhanced nervous activity in the remaining pathway

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D 19

Oxygen a Coronary Vasoconstrictor

By FLEMMING BONDE PETERSEN and R JAMES BARNARD *August Krogh Institute University of Copenhagen Denmark and Division of Thoracic Surgery University of California Medical Center Los Angeles USA*

Seven mongrel dogs had a flow probe placed around the left coronary artery and a pressure transducer placed in the wall of the aortic arch Two weeks after the operation resting values were measured during breathing of either 21 % or 100 % O_2 administered from pres-

through a mask fitted around the dog's nose The dog breathed alternating 1[?] % 100% 21% O_2 for each 6 min periods Measurements were recorded on a every 2nd min for coronary blood flow (COF) and aortic pressure (AP) From res mean diastolic CBF and mean diastolic AP were computed by planimetry ng 21% was 31 7±5 7 (SE) ml min and decreased significantly (p<0 05) to (SE) ml min⁻¹ Conductance (CBF AP) decreased (p<0 05) from 460± 078 073 ml min mmHg during 21% and 100% O_2 respectively Oxygen delivery eral O_2 content) remained unaffected. It is concluded that in the normal dog cts as vasoconstrictor autoregulating CBF according to O_2 needs.

D 20

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	Blood citrate. Fat diet	µmol/l Carb diet	Blood pyruvate. Fat diet	µmol/l Carb. diet
st				
ercise 5 min	58.3±13 1	51 4±13.3	31.9± 7 7	70 4±24 ***
xercise 23 min	58.8±12.8	53.5±13.6	136 ±19	164±27*
	74 1±18 0	66 4±17.6**	143 ±28	181±43
t	−14.3± 5.0	−12.5± 4 1	+4.3± 4.8	+8.4±12
rcise 5 min	−3.2± 1 5	−1 6± 1.0*	−16.7± 8.3	−6.5±10*
rcise 23 min	−3.2± 1.8	−4.0± 2.2	−5.8±12	−4.8±11

are mean ±SD Levels of significant difference between fat and carbohydrate rich diet are: (*), p<0 01 (**) and p<0 001 (***).

pyruvate and lactate at rest, were not different between diets After 23 min of exercise there were no differences in citrate and pyruvate release but lactate release was much higher after carbohydrate However after 5 min of exercise there were greater release of citrate and pyruvate and lower release of lactate after fat than carbohydrate diet (Table 1). This might indicate that high levels of FFA at onset of exercise leads to increased synthesis of citrate increasing the level of acetyl-CoA Increased pyruvate release in spite of low lactate release indicates decreased pyruvate oxidation rate

In conclusion the results are consistent with the theory that increased oxidation of fatty acids elevate citrate concentration thereby inhibiting glycolysis and pyruvate oxidation.

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D 21

A Study of Thalamo-cortical Recruiting Responses during the Reserpine State in the Cat

By A LAIHINEN and P VALLEALA *Department of Physiology University of Turku Finland*

In this study the state induced with reserpine—reserpine state—is considered an entity of its own as contrasted against wakefulness slow wave sleep (S) and paradoxical sleep (PS) Reserpine in a dose of 0 50 to 0 75 mg/kg induces in the cat a longlasting sedation and ponto-geniculo-occipitale wave activity (PGO) which normally occurs during PS and the final phase of S immediately preceding PS In this study the "early" reserpine state is characterized by electrocortical desynchronization, PGO activity with stereotype rhythm and moderate neck muscle tone and the "late" reserpine state by temporary synchronization of electrocortical activity and the first signs of PS S and PS do not occur during the early reserpine state whereas the late reserpine state means the gradual restitution of normal sleep

Thalamo-cortical recruiting responses (RRs) most typically encountered in barbiturate anesthesia and during S can be elicited in a modified form during PS Recently it was shown that RRs do not usually occur simultaneously either with PGO waves during PS (PGO_{PS}) or with PGO waves in the early reserpine state (PGO_{RES}) (Laihinen and Valleala 1977) In the present study the relationship of RRs and PGO_{RES} was further analyzed in the late reserpine state.

Serpasil® was given 0 75 mg/kg/i p PGO_{RES} waves were recorded in the geniculate nuclei. The centre median nucleus was stimulated at a rate of 7–9 Hz. The RRs were recorded from the anterior sigmoid gyrus. During the late reserpine state when the electrocortical activity was synchronized there were plenty of PGO_{RES} waves not only in the presence of spontaneous spindles but also during RRs i e. the mutual exclusion found in

the early reserpine state did not exist. When PS reappeared that exclusion could be observed.

Normally spontaneous spindle and PGO_{PS} activity can occur simultaneously during the transition from S to PS. This is analogical to the coincidence of spindle-like activity (spontaneous spindles and RRs) and PGO_{RRS} waves during the late reserpine state. In both the situations neither PS nor S has an absolute dominance. It is a dynamic equilibrium with certain components of both S and PS-spindle activity and PGO waves even simultaneously and could be related to the changing concentrations of the relevant transmitters.

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D 22

Morphology and Function of M. Biceps Brachii in Man. A Worth-while Experimental Model?

By E. NYGAARD, K. JORGENSEN, M. HOUSTON, J. SUZUKI and B. SALTIN. *August Krogh Institute, University of Copenhagen, Copenhagen, Denmark*

Skeletal muscle of most species comprise fibres with different characteristics. A subdivision in one slow (ST) and two fast twitching (FT) fibres is usually satisfactory. A close coupling between histochemical and biochemical characteristics of the various fibre types and their function has been established in muscles from rat, guinea pig and cat (for ref. see Burke and Edgerton, 1975).

In skeletal muscle of man three fibre types have also been described, although the differences between the two fast twitching fibres (FTa and FTb) has not yet been established (for ref. see Saltin *et al.* 1977). Indirect evidence for a coupling between muscle fibre characteristics and function of skeletal muscle of man are available from studies of glycogen depletion pattern, and of single motor unit recordings (Grimby and Hannertz 1977). Further Thorstensson (1976) using an isokinetic device and knee extension has found certain relationships between fibre composition of the vastus lateralis and certain capacities of the thigh muscle. However, there are some limitations to this model, one being that different muscles are involved in the knee extention and another that less than half the maximal velocity can be achieved.

To overcome some of these difficulties we have chosen to use a modification of Hill's method to establish the force velocity relationship for arm flexion in six adult healthy subjects. Muscle biopsies were obtained from the central part of the biceps brachii muscle for histochemical and biochemical analysis. Estimation was made of the cross sectional area of the muscle mass. To evaluate endurance capacity dynamic work was performed to exhaustion at 20% MVC.

Results: Fibre distribution averaged 45% St fibres (range: 14–63%), 27% FTa (14–34%) and 28% FTb fibres (13–57%). Fibre distribution did not correlate with any functional parameters. Mean fibre area correlated with cross section of the muscle mass as well as

with MVC (p<0 01) the correlation being most pronounced between mean area of F fibres and MVC (r=0 88 p<0 001) Total FT area of the muscle correlated with streng at 50% of maximal velocity as well as with velocity at 50% MVC (p<0 01) the FTa fibr showing a higher correlation than the FTb fibres No relationship was found between tl oxidative characteristics (proportion of ST fibres relative ST area, capillarization ar SDH activity) and endurance capacity of the muscle (performance time total work rise oxygen uptake and heart rate)

In conclusion it can be stated that significant relationships have been found betwee some fibre characteristics of the biceps brachii and its function indicating that the stuc of this muscle during arm flexion may well serve as a good experimental model The lac of relationship between certain variables is most likely due to a relatively large variabili in the measurement of some of these parameters in comparison to the rather narrow rang for these variables found in the few subjects studied

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D 23

Simultaneous Measurements of Capillary Diffusion and Filtration Exchange at Alterations in the Capillary Permeability-Surface Area Product (PS) and During Shifts in Filtration-Absorbtion

By B RIPPE A KAMIYA and B FOLKOW *Department of Physiology University of Göteborg Sweden*

Only seldom has it been possible to compare quantitatively and simultaneously capillary diffusion and filtration events in well defined vascular beds during various situations Therefore the diffusion exchange of Cr EDTA measured with a colorimetric variant of the single injection indicator diffusion method (Rippe and Stage 1976) was followed simultaneously with estimations of the capillary filtration capacity (CFC) using the "isogravimetric" rat hindquarter preparation during artificial perfusion and maximal dilatation. Measurements were performed at constant flow and during 1) alterations of permeability (i.e. infusion of histamine) 2) alterations of perfused capillary wall area (graded rarefication of the capillary network by microspheres Ø~15 μm) and 3) shifts in filtration-absorption

At maximal vasodilatation during "control" CFC was 0 037±0 001 ml/min×mm Hg ×100 g and PS for Cr EDTA was 5 67±0 13 ml/min×100 g. Histamine increased CFC nearly fourfold while PS for Cr EDTA remained almost unchanged In contrast alterations in capillary wall area, as reflected by roughly reciprocal changes in flow resistance

from control, resulted in proportional changes in PS for Cr EDTA while the CFC changes were always relatively smaller. Transcapillary solute transfer from vessels to tissue was only little affected by shifts in capillary filtration-absorption. During filtration PS for Cr EDTA increased only slightly reaching 6–7% increase at a filtration rate of 1 ml/min×100 g. During absorption the capillary to tissue transfer of Cr EDTA remained essentially unchanged.

The present findings may suggest that 1) histamine increases capillary permeability by increasing the number and/or mean radius of the large pores which because of their relative paucity are of little importance for small molecular diffusion exchange but, by virtue of their large radius, are highly important for convective and macromolecular exchange. ... lterations of the perfused capillary wall area are closely reflected by changes in PS ... ct, but somewhat underestimated by CFC measurements since CFC estimations are ... as "flow-limited" 3) Blood to tissue diffusion of small molecules is only little modified by convection effects, but this is not necessarily true for back diffusion.

...pported by grants from the Swedish Medical Research Council No B78-14X-0016- ...nd from the Medical Faculty University of Göteborg.

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D 24

...cteristics of Cardiac Vagal Afferents in Normotensive and Hypertensive Rat

By P. THORÉN, S. E. RICKSTEN and E. NORESSON *Department of Physiology, University of Goteborg, Goteborg, Sweden*

...l receptors with vagal afferents were first described by Paintal and has been studied ...tail in dogs and cats (Paintal 1973). The heart of cats and dogs also has a large population of atrial (Thorén 1976) and ventricular receptors (Thorén 1977) with non-medullated ...rats.

...e aim of the present study was to examine the characteristics of the vagal cardiac ...ents in normotensive and hypertensive rats. The rats were anaesthetized with nembu... in the dose of 50 mg/kg b.w. intraperitoneally. Thorax was opened and a catheter were ...ed in the left atrium via a lung vein. The aortic pressure was measured via a femoral ...eter. Snares were placed round the ascending aorta and the pulmonary artery. Filaments were dissected from the right vagus and tested for activity coming from the heart. ...dissections were continued until adequate single fiber recordings were obtained. ...ormotensive rats: Recordings were obtained from 13 receptors in 9 rats. 11 receptors ...located to the left atrium and 2 receptors to the right atrium. No receptors were found ...e ventricles. The conduction velocities were 0.6–1.3 m/sec. No receptors with medul-

lated afferents were found Threshold for activated of the left atrial receptors were 2.5–11.5 mm Hg in mean left atrial pressure and maximal frequencies varied between 8 and 55 Hz 10 of 13 receptors showed a clear cardiac rhythmaly upon activation

Hypertensive rats Recordings were obtained from 4 left atrial receptors in 4 spontaneous hypertensive rats These receptors have thresholds of 9–12 mm Hg.

Conclusion. The cardiac vagal receptors in the rat seem to be located mainly in the atria and are connected to afferent non-medullated afferent nerve fibers Preliminary experiments indicates a higher threshold of these receptors in the hypertensive rat

Supported by grants B77 14X-04764 and B76-14X-0016-14A from Swedish Medical Research Council

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D 25

Sodium Balance During Development of Hypertension in Spontaneously Hypertensive Rats (SHR)

By S LUNDIN H HERLITZ G BERGLUND S E. RICKSTEN G GÖTHBERG and M HALLBÄCK. *Departments of Physiology and Med Clin I University of Göteborg Göteborg Sweden*

The mechanisms leading to primary hypertension in man and in SHR are still poorly understood SHR display signs of enhanced sympathetic activity and plasma volume is slightly lowered (Rippe Lundin and Folkow 1977) but in the Milan hypertensive strain of rats (MHS) the pressure rise is associated with sodium retention and plasma volume increase (Bianchi *et al* 1975) With increasing severity of hypertension man displays a decreasing ability to excrete salt and water and an increasing renal resistance (Berglund *et al* 1977) Thus both MHS and man display signs of an alteral renal handling of sodium initially or along the course of hypertension and the question arises whether also SHR show similar alterations of either primary or secondary nature

A comparison was therefore performed concerning sodium handling in SHR and normotensive control rats (NCR) primary in early phases of SHR hypertension. The rats were kept in metabolic cages and sodium intake urinary and faecal sodium excretion plasma renin activity and urinary aldosterone excretion were determined at the age of 6 weeks 3 and 7 months Constant flow perfusions of the kidneys were also performed to determine total renal vascular resistance in the different age groups

At six weeks of age fractional urinary excretion (urinary excretion/intake × 100) in SHR (29±2%) was lower ($p<0.001$) than in NCR (57±1%) while fractional faecal excretion was higher ($p<0.001$) in SHR (27±2%) than in NCR (12±1%) Total fractional excretion

(urinary + faecal excretion/intake × 100) was significantly lower in SHR than in NCR (56 ± 2 vs 68 ± 1%) but a true difference in sodium retention (μmol Na/g body weight increase) could not be demonstrated due to a correspondingly lower Na intake in SHR. Urine aldosterone activity was slightly higher in SHR (15 per cent). The same excretion pattern was found at 3 months of age although total fractional excretion no longer differed significantly and at 7 months also urinary excretion was largely equal in SHR and NCR. The renal vascular bed showed to be progressively more structurally rebuilt with age.

The results do not suggest any initial sodium retention in SHR, in agreement with reduced plasma volume but the routes of sodium elimination are shifted from kidneys towards gastro-intestinal tract. This can hardly be explained by the observed slight changes in aldosterone but neurogenic hormonal and structural alterations of renal resistance and the pre/postglomerular resistance ratio (Folkow *et al.* 1977) might be involved.

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D 26

...ma Volume, Blood Volume and Transcapillary Escape Rate (TER) of Albumin in Young Spontaneously Hypertensive Rats (SHR) Compared with Controls (NCR)

By B. RIPPE, S. LUNDIN and B. FOLKOW. *Department of Physiology, University of Göteborg, Sweden*

It has recently been discussed whether a "primary" blood volume increase may be a common cause of hypertension, by enhancing venous return and cardiac output which, in turn, could initiate a sustained resistance increase by "whole body autoregulation" (*e.g.* Guyton *et al.* 1971). Such a chain of events may, indeed, be an important initiating element in the Milan spontaneously hypertensive rat (MHS) (Bianchi *et al.* 1975). In SHR, on the other hand, central neurohormonal influences appear to constitute a major initiating element. The question arises whether an early volume increase might contribute also to SHR hypertension.

Plasma and blood volumes were therefore compared in groups of young SHR and NCR, particularly during the phase of rapid pressure rise in SHR (3, 4, 5, 6, 8, 10 weeks of age and 17 months of age). Also TER was measured in 6–10 week old SHR and NCR, since TER is commonly increased in man's primary hypertension (Ulrych 1973). After i.v. in-

lated afferents were found Threshold for activated of the left atrial receptors were 2.5–11.5 mm Hg in mean left atrial pressure and maximal frequencies varied between 8 and *55 Hz. 10* of *13* receptors showed a clear cardiac rhythmaly upon activation.

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D 25

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spatial frequency and the size of the gratings were scaled by the inverse of the cortical ...ification factor estimated from the points which Cowey and Rolls calculated from the ...a of Brinley and Lewin (1968) who mapped the phosphenes caused in the lower nasal ...al field by electrical stimulation of human visual cortex. This way the cortical repre-...ations of the gratings were presumably made similar at various eccentricities. For a ...ies of eccentricities of 0, 1.5 4 7.5 14 and 30 deg the following values of magnification ...tor were assumed. 7 75 5.25 3 44 2.31 1.24 and 0.49 mm/deg. The scaling of gratings ...the inverses of these values was accomplished by keeping the gratings constant and ...ving the subject closer to the display The CSFs recorded with scaled cortical repre-...ations were almost identical.

Our result implies that a picture can be made equally visible at any eccentricity by scal-...its size by the magnification factor for the CSF represents the spatial modulation ...sfer function of the visual system for near-threshold contrasts. The constancy of the ...topic CSFs of different but equally large regions of visual cortex indicates a striking ...ctional homogeneity of image processing machinery

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D 28

...dividual Blood Lactate Response during Exercise and Its Relation to Muscle Fibre ...position

By P K. PEDERSEN, *Inst of Physical Education, Odense University Denmark*

...he general pattern of the blood lactate concentration (HLa) during graded exercise is an ...altered or slightly increased level during light and moderate exercise followed by a steep ...se during severe exercise. Although this is the common pattern, considerable individual ...fferences exist between HLa values at higher relative work loads (percentage of V_{O_2}max) Studies on muscle fibre composition on humans and animals have shown that muscles ...e composed by fibres that differ in mechanical as well as biochemical (oxidative–glyco-...tic) respects. In order to find out whether variations in muscle fibre composition could ...ccount for the individual differences in lactate response to exercise, 17 trained subjects ...ad biopsies taken from the rectus femoris muscle Based on histochemical staining the ...uscle fibres were classified in types I, II A and II B Type I is considered to be high ...oxidative-low glycolytic, type II A oxidative-glycolytic and type II B low oxidative-high ...glycolytic (Essén *et al* 1975).

The blood lactate response was studied in a progressive bicycle exercise test comprising ...3-5 submaximal work intensities followed by a maximal test. The measurements included ...oxygen uptake as well. From these data individual relative load-blood lactate curves were

jections of I^{125} labelled human serum albumin blood samples were withdrawn 20 min later for determination of hematocrit and with estimation of blood and plasma volumes from the extent of tracer dilution TER was estimated from the gradual reduction with time of the tracer concentration in plasma.

The results show that during early age plasma volume was slightly but significantly ($p<0\ 001$) lower in all the SHR groups when compared with NCR at identical body weights Though hematocrit was slightly higher in SHR than in NCR also blood volume tended to be lower in SHR ($p=0\ 06$) First at the age of 7 months when SHR hypertension is fairly advanced with beginning complications blood and plasma volumes tended to be higher in SHR than in NCR. TER was increased in SHR 19 7±0 8 per cent/hour compared with 16 4±0 7 in NCR ($p<0\ 01$)

These results suggest that SHR hypertension unlike that in MHS is not dependent of any "primary" increase in cardiovascular filling. The reduced blood volume hemoconcentration and increased TER is in agreement with the view that an early enhancement of sympathetic activity is a dominant initiating element in SHR hypertension as this will somewhat raise also venous and capillary pressures in SHR.

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D 27

Cortical Representation of Visual Field predicts the Photopic Contrast Sensitivity of Human Peripheral Vision

By JYRKI ROVAMO VEIJO VIRSU and RISTO NASÄNEN *Institute of Physiology and Department of General Psychology University of Helsinki Finland*

Hilz and Cavonius (1974) found that photopic contrast sensitivity of human vision decreases as the gratings are moved farther away from the fovea. Similarly the area of visual cortex devoted to the analysis of a constant size region in the visual field diminishes progressively for more peripheral locations (Cowey and Rolls 1974) In the following we shall provide evidence that decrease of sensitivity is due to diminished cortical area

We measured the spatial contrast sensitivity functions (CSFs) at various eccentricities along the nasal and inferior meridians of the visual field by finding the inverses of threshold contrasts for a series of sinusoidal gratings In this experiment the semicircular grating with 8 cm radius was viewed at a constant distance of 458 cm Eccentricity refers to the angular distance between the fixation point and the nearest edge of the grating At increasing eccentricities both contrast sensitivity and maximal resolution of high-contrast gratings decreased radically In the second experiment the eccentricities were the same but both

t 1. Adipose tissue blood flow (ATBF) plasma glycerol and core temperature during exercise and induced hyperthermia

	Control	I	II	III	IV	Control
ise						
t. ATBF 100 g⁻¹ min⁻¹	1 4 (0 9)	4.0 (1.5)	4.3 (2.1)	5.8 (2.9)	4.8 (2.3)	_.8 (2.3)
nal ATBF 100 g⁻¹ min⁻¹	2.3 (3.2)	4.3 (2.4)	8.1 (4.8)	12.8 (6.3)	15 4 (13.2)	8.1 (3.8)
na glycerol l l⁻¹	86 (23)	244 (11)	380 (74)	495 (90)	579 (118)	375 (180)
temperature	36.9 (0.3)	37.8 (0.4)	37.8 (0.2)	37.6 (0.3)	37.3 (0.5)	36.5 (0.2)
rthermia						
t. ATBF 100 g⁻¹ min⁻¹	2.3 (1.5)		3.5	(1.2)		1.8 (1.3)
na glycerol l l⁻¹	77 (11)		95	(28)		105 (16)
temperature	<36.9		38.5 (0.3)			37 1 (0.3)

jects participated in each experimental series. Mean values are given. Figures in brackets are ard deviations.

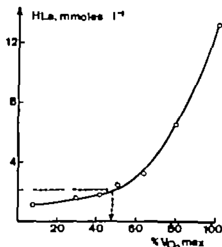
To study the possible role of the increased subcutaneous ATBF in the regulation of temperature, 2 series of experiments were carried out: *A* experiments comparing taneous and perirenal ATBF during exercise based on the thought that a possible ase in perirenal ATBF hardly could serve a thermoregulatory purpose. *B* Measurements of subcutaneous ATBF during passively increased body temperature

ethods Subcutaneous ATBF was measured as described in (1) Perirenal ATBF was sured by 127 Xe wash-out from the infrarenal fat tissue after injection guided by ultrad. In series A the subjects worked at 50% maximal O_2-uptake for 4 consecuti e 50 ute periods, separated by 10 minute rest intervals. In series B the body temperature raised about 1.5°C by a hot bath and maintained by stay at an ambient temperature 7°C, humidity 70% Core temperatures were measured by telemetry using a thermo swallowed by the subject.

esults and discussion. The experimental findings are given in table I The increase in cutaneous ATBF during exercise was confirmed. However the perirenal ATBF inased even more (the increase significant at the 2 percent level) No significant rise in BF was seen during passive rise of the core temperature It is concluded that the rise ATBF during exercise does not serve thermoregulatory purposes. The increase in ATBF he two tissues studied correlates well with the intensity of lipolysis as evaluated by plasma glycerol concentrations.

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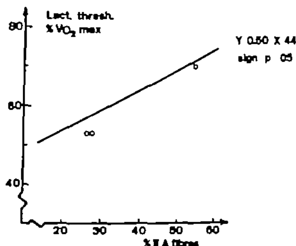


Fig. 1 A. Individual work load – blood lactate curve. The dotted line and the arrow indicate the establishment of the lactate threshold in this case 48 % of Vo_2max. B The relationship between the percentage of type II A fibres and the lactate threshold (N = 17)

constructed (Fig. 1 A) and used to determine a so-called lactate threshold (arbitrarily defined as the load in % of V max which is associated with HLa = 2.2 mM) The range of lactate thresholds was 42–81 % V_{O_2}max When correlating the lactate thresholds with the percentage values for the different fibre types a significant relationship was observed for type II A only (Fig. 1 B)

It is known that HLa at a given relative load decreases after training. Further after training the number of type II A fibres increases at the expense of type II B (Andersen & Henriksson) The results of the present study suggest that the two phenomena are causally related.

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D 29

Subcutaneous and Perirenal Adipose Tissue Blood Flow during Prolonged Exercise in Man

By J BÜLOW and J MADSEN *Institute of Medical Physiology C University of Copenhagen Denmark*

It has previously been shown (1) that subcutaneous adipose tissue blood flow (ATBF) in man increases 3–4 fold during prolonged exercise The purpose of the present experiments

ight (BW-TBW) and increased their metabolic rate while some subjects responded posite. The individual subjects showed the same pattern during semistarvation i.e. ber a decrease in the metabolic rate and a constant weight or vice versa. The changes metabolic rate in each subject were of the same magnitude regardless of the conditions rest or work, before or after a meal. The observed change in metabolic rate if supposed to be present all 24 hours of the day only accounts for about one tenth of the increased or decreased dietary intake in the subject with constant weight. The experiments e no explanation for this discrepancy

D 31

The Effects of Glucagon and Secretin on Bile Production in Cats

By N Kraglund and J A Larsen *Institute of Physiology University of Aarhus*

Although similar in structure the biological effects of glucagon and secretin differ in many ways. In the present experiments the effects of the two hormones on bile production and liver metabolism were studied in fasting cats anaesthetized with chloralose. Bile was collected from the choledocus and the biliary loss of bile acids was compensated for by continuous infusion of taurocholate. The canalicular bile production was estimated by the biliary clearance of ^{14}C-erythritol. After a control period of 60 min glucagon (0 1 $\mu g/kg/$min) or secretin (2 $\mu g/kg/h$) was infused. After a 45 min delay glucagon increased bile flow by 30% due to an increase in canalicular bile production. The bile acid excretion rate remained constant and HCO_3^--excretion rate increased in parallel with bile flow. Infusion of secretin was immediately followed by a 84% increase in bile flow caused by a change from net reabsorption to net secretion of fluid in bile ductuli. The bile acid excretion rate was not affected but in contrast to glucagon secretin caused a 156% increase in the excretion rate of HCO_3^-. It thus appears that although both hormones do increase bile flow the mechanism of action is quite different, glucagon affecting the non-bile-acid dependent canalicular bile production and secretin stimulating the bile ductuli secretion of a bicarbonate rich fluid

D 32

β_2-adrenoceptors Mediate the Stimulating Effect of Adrenaline on Active Electrogenic Na K-transport in Rat Soleus Muscle

By J A. Flatman and T Clausen *Institut of Physiology University of Aarhus DK-8000 Århus C Denmark*

It has been known for a long time that catecholamines (CA) can hyperpolarize skeletal muscle fibers (see Bowman and Nott 1969) Tashiro (1973) demonstrated that isoprenaline increases the membrane potential in guinea pig soleus muscle, and that this effect was blocked by ouabain.

D 30

Energy Expenditure and Weight Responses to Overeating and Semistarvation in Man

By O LAMMERT A G MØLGAARD E S HANSEN and M G RASMUSSEN *Inst for Physical Education Odense University Denmark*

The effect of a variation in the dietary intake on the oxygen uptake the respiratory quotient the body weight and total body water was investigated The results were compared with measurements obtained during a preceding period with a normal habitual food intake Before and after a testmeal (4 MJ) V_{O_2} and V_{CO_2} were measured at rest and at work (36 W) The weight of the dry body matter was determined from the body weight (BW) and determination of total body water (TBW) (tritium)

Measurements were performed in nine normal subjects once a week while dietary energy intake was normal and habitual and while it was increased to 24 MJ per day for two weeks. Four of the subjects continued another two weeks with an intake of 2 1 MJ per day The daily dietary intake was weighed and the energy content was determined using tables The results are shown in figure 1 During overeating some subjects kept a constant

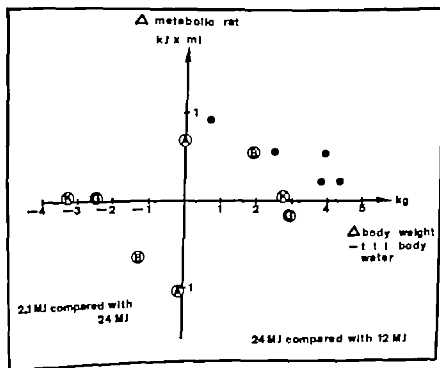


Fig. 1 Changes in energy expenditure in nine normal subjects compared with their changes in dry body matter (body weight – total body water) The right side of the diagram shows the changes obtained when the normal period (12 MJ per day) was compared with the overeating period (24 MJ per day).

The left side of the diagram shows the changes obtained on four of the nine subjects when the overeating period (24 MJ per day) was compared with the semistarvation period (2.1 MJ per day)

The letters (A–B–G & K) are used to identify the subjects in the two situations

In cats an ileal segment was isolated with intact nervous and vascular supply and with the ends outside the abdominal wall (Thiry Vella loop) After recovery from surgery the animals were subjected to acute experiments under chloralose anesthesia. The vagi were cut in the neck and stimulated in the efferent direction to ensure a constant and known submaximal level of cholinergic tone in the stomach, the volume of which was recorded by a large balloon inserted via the esophagus. The spinal GI–GI reflex could be repeatedly elicited by distension of the isolated Thiry Vella loop, which elicited a prompt increase in gastric volume With this preparation it was thus possible to study sympathetically mediated effects induced by hypothalamic stimulation in the presence or virtual absence of spinal GI–GI reflex influences on gastric motility

Systematic topical stimulations of the hypothalamus revealed an area which could abolish the inhibition of stomach motility caused by the GI–GI reflex, but without affecting gastric tone if this reflex was not present. In addition, stimulation of this area, which appeared to coincide with the hypothalamic sympatho-inhibitory area described by Folkow Johansson and Öberg (1959) regularly caused decreases in arterial blood pressure and heart rate It is concluded that there are well-defined hypothalamic sympatho-inhibitory neuron pools, which exert an inhibitory influence on the spinal GI–GI reflex, in association with a widespread inhibition of sympathetic discharge to the cardiovascular system

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D 34

Insulin and Bile Production in the Cat

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Previous experiments have demonstrated that administration of insulin stimulates that part of the canalicular bile production which seems to be dependent on the activity of Na-K ATPase (Larsen and Christensen 1977) Whether this effect of insulin is a direct effect of the hormone or secondary to metabolic changes caused by insulin is not established In order to examine if insulin actually comes into contact with the canalicular membrane the biliary excretion of insulin was examined The experiments were performed on 6 fasting cats anesthetized with chloralose Insulin (Insulin Leo® Neutral Nordisk Insulin Lab) was given i. in doses ranging from 0 15 U/kg to 1 U/kg. Bile was collected from the choledocus and the loss of bile acids compensated for by infusion of taurocholate 0.2 μmole/kg/min. Canalicular bile flow was estimated by the biliary clearance of C^{14}-erythritol. Insulin in arterial and portal venous blood and bile was determined by a double-antibody technique using Wick chromatography (Ørskov Thomsen and Yde 1968)

In a combined electrophysiological and ionic flux study we have shown that in the isolated rat soleus muscle CA at concentrations down to the physiological level induce a hyperpolarization which is presumably the result of stimulation of active coupled Na K -transport (Clausen and Flatman 1977) Both actions were potentiated by theophylline and could be mimicked by dibutyryl-cyclic AMP The effects of adrenaline were blocked by the β_1-β_2-adrenoceptor antagonist propranolol but were unaffected by the α-adrenoceptor antagonist thymoxamine Moreover as the specific β_2-adrenoceptor agonist salbutamol was found to be a potent stimulator of active electrogenic Na K transport, we concluded tentatively that these actions of CA were mediated by β_2-adrenoceptors.

We have now found that adrenaline is at least 100 times more potent than the specific β adrenoceptor agonist H 133/22 at stimulating active Na-extrusion and evoking hyper polarization in rat soleus muscle In the heart, adrenaline is 10 times more potent than H 133/22 at stimulating β_1-adrenoceptors (E. Carlsson pers. comm) Propranolol (10^{-7} 10^{-6} M) is at least 10 times as potent as the specific β_1-adrenoceptor antagonist metoprolol at inhibiting the action of adrenaline (10^{-7} 10^{-6} M) on ^{22}Na-efflux and suppressing the hyperpolarizing effect of adrenaline As propranolol and metoprolol are nearly equipotent at blocking β_1-adrenoceptors (Åblad et al 1973) our results provide further support for the idea that the effect of CA on the electrogenic Na^+ K transport in rat soleus muscle is mediated via β_2-adrenoceptors

We thank Drs. B Åblad and E. Carlsson of AB Hässle for stimulating discussion and the gift of metoprolol and H 133/22

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D 33

Hypothalamic Inhibition of the Spinal Gastrointestino-Gastrointestinal Reflex

By D Delbro K. Bongsell and B Lisander. *Department of Physiology University of Göteborg Sweden*

Both motility and circulation in the gastrointestinal tract are modulated by the gastrointestino-gastrointestinal (GI-GI) reflex whose efferent link is the regional adrenergic outflow This reflex can be elicited by distension of the gastrointestinal tract or by nociceptive stimuli within the abdominal cavity Although basically spinal it is subject to a supraspinal modulation which is net inhibitory in nature (*cf* Furness and Costa 1974) The present experiments have been performed to elucidate whether this reflex can be influenced from specific areas of the hypothalamus

a found that 6-OHDA treatment caused considerable reduction of the adrenergic but single terminal axons did persist between the two media layers. No marked in muscular dimensions was observed Functionally some phasic spontaneous was recorded in all preparations although in sympathectomized tissues it did not throughout the experiments. The sensitivity to exogenous noradrenaline (NA) increased in 6-OHDA treated vessels and the maximum response was greater than als. Maximum responses to transmural nerve stimulation (NS) were reduced when ed as a percentage of the maximum NA response (59±3.5 *v.s* 89±1.5 per cent n=6). Furthermore significant responses to NS were only obtained at high "unogical" impulse rates in 6-OHDA treated animals.

concluded that neonatal 6-OHDA treatment leads to marked but incomplete symomy Growth and morphological differentiation of the media of the portal vein did n treated animals but some functional changes were observed. Apparently no ef vasomotor nerve control persisted. This would indicate either that the sparse re- adrenergic supply had been sufficient to govern ontogenesis or that development olled by other mechanisms.

orted by Swedish Medical Research Council 2207 4173 3884 Magn. Bergvall's tion and the Medical Faculty

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D 36

ptic β-adrenoceptor Mediated Facilitation of Noradrenaline Release in Rat Portal

C. DAHLÖF B LJUNG and B ÅBLAD *Dept of Pharmacology AB Hässle Mölnl, Sweden*

d superfused portal veins of spontaneously hypertensive rats (SHR) were used in mpt to quantify the importance of a presynaptic β-adrenoceptor mediated feedback tion for neuronal noradrenaline release and effector response (Adler-Graschinsky 974 Dahlof *et al.* 1975 Stjärne *et al.* 1975). The veins were preincubated in Krebs n containing (8.5×10^{-7} M) ^{3}H 1-noradrenaline (NA) at +37°C. After rinsing, the vein preparation was mounted in an organ bath with continuous flow of Krebs n (1.5 ml×min $^{-1}$) at a passive tension of 5 mN Muscle tension was recorded conly and the effector response (ER) was determined as integrated isometric force. perfusate was collected in 4 min fractions during periods of spontaneous activity eurogenic responses to transmural field stimulation (TNS) with trains of 240 pulses z. Three stimulation periods S1 S2 and S3 were performed with 20 min intervals. es in fractional release of tritium per shock (Af) and ER during S3 expressed as a

In accordance with previous experiments insulin increased canalicular bile flow up to 63 % without affecting the excretion rate of bile acids which was 0.30±0.03 umole/kg/min before insulin and 0.30±0 02 umole/kg/min after insulin The insulin concentrations in arterial and portal venous blood were almost identical and after reaching a peak concentration ranging from 235 to 5 000 uU/ml they decreased exponentially within 30–90 min. Insulin appeared in bile with a delay of about 15 min with peak concentrations ranging from 40–210 uU/ml and the exponential decay lasted from 30 to 120 min. The occurrence of insulin in bile was always accompanied by a rise in bile flow and changes in the biliary concentration of insulin were often followed by parallel changes in bile flow In general the insulin concentrations in bile returned to control values before bile flow had normalized

In conclusion the experiments have demonstrated that insulin is able to pass the canalicular membrane and the possibility therefore exists that the choleretic effect of insulin may be a direct effect of this hormone on the canalicular ATP-ase system

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D 35

Neuroeffector Development in the Rat Portal Vein after Neonatal Treatment with 6-hydroxy dopamine

By B LJUNG J LUNDBERG and A DAHLSTRÖM *Department of Physiology and Institute of Neurobiology University of Göteborg Sweden*

During the first few postnatal weeks the media of the rat portal vein becomes divided into a heavy outer longitudinal and an inner circular layer A terminal adrenergic nerve plexus forms between the muscle layers (Lundberg *et al* 1976) Correlated to this morphological maturation functional properties develop in sequence During the third week of life phasic spontaneous activity and responses to nerve stimulation at low frequencies appear *i.e* characteristic features of the adult portal vein (Ljung and Stage 1975)

With the aim of elucidating the role of the adrenergic innervation for the ontogenesis of propagating vascular muscle we have studied effects of adrenergic denervation induced by intense 6-hydroxydopamine (6-OHDA) treatment on morphological and functional development of the rat portal vein.

Newborn Sprague Dawley rats were treated with 6-OHDA (100–250 mg/kg s c) dissolved in saline with ascorbic acid or with ascorbic acid solution repeatedly during the first two postnatal weeks At 4 weeks of age the rats were sacrifized and isolated portal veins were either studied directly by the fluorescence histochemistry method of Hillarp and Falck or mounted in an organ bath for isometric recording of contractile activity of the longitudinal muscle and thereafter studied histochemically as stretch preparations.

...un found that 6-OHDA treatment caused considerable reduction of the adrenergic ...s but single terminal axons did persist between the two media layers. No marked ...e in muscular dimensions was observed. Functionally, some phasic spontaneous ...y was recorded in all preparations although in sympathectomized tissues it did not ...t throughout the experiments. The sensitivity to exogenous noradrenaline (NA) ...d increased in 6-OHDA treated vessels and the maximum response was greater than ...trols. Maximum responses to transmural nerve stimulation (NS) were reduced when ...ssed as a percentage of the maximum NA response (59 ± 3.5 v.s. 89 ± 1.5 per cent ...n=9). Furthermore, significant responses to NS were only obtained at high "un-...logical" impulse rates in 6-OHDA treated animals.

...s concluded that neonatal 6-OHDA treatment leads to marked but incomplete sym-...ctomy. Growth and morphological differentiation of the media of the portal vein did ...in treated animals but some functional changes were observed. Apparently no ef...e vasomotor nerve control persisted. This would indicate either that the sparse re-...ing adrenergic supply had been sufficient to govern ontogenesis or that development ...trolled by other mechanisms.

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D 36

...aptic β-adrenoceptor Mediated Facilitation of Noradrenaline Release in Rat Portal

By C. DAHLÖF, B. LJUNG and B. ÅBLAD, Dept. of Pharmacology, AB Hässle, Möln-dal, Sweden

...ed superfused portal veins of spontaneously hypertensive rats (SHR) were used in ...tempt to quantify the importance of a presynaptic β-adrenoceptor mediated feedback ...anism for neuronal noradrenaline release and effector response (Adler-Graschinsky ... 1974, Dahlöf et al. 1975, Stjärne et al. 1975). The veins were preincubated in Krebs ...tion containing ($8.5 \times 10^{-}$ M) ^{3}H-1-noradrenaline (NA) at +37°C. After rinsing, the ...al vein preparation was mounted in an organ bath with continuous flow of Krebs ...tion (1.5 ml × min $^{-1}$) at a passive tension of 5 mN. Muscle tension was recorded con-...ously and the effector response (ER) was determined as integrated isometric force. ...superfusate was collected in 4 min fractions during periods of spontaneous activity ...neurogenic responses to transmural field stimulation (TNS) with trains of 240 pulses ... Hz. Three stimulation periods S1, S2 and S3 were performed with 20 min intervals. ...nges in fractional release of tritium per shock (Δt) and ER during S3 expressed as a

percentage of those obtained during S2 were used to evaluate the effects of the test substances which were introduced 16 min before S3

It was found that tetrodotoxin (8×10^{-7} M) abolished both Δt and ER and phenoxybenzamine (10^{-6} M) abolished ER. Together these findings indicate that ER was due to neuronically released NA

Phenoxybenzamine produced an almost 5 fold increase of Δt probably mainly due to inhibition of presynaptic alpha receptors mediating a negative feedback of neuronal NA release (Starke 1972)

Isoprenaline (3×10^{-8} M) increased Δt by 12±3 per cent ($p<0.05$) but did not affect the ER.

dl propranolol (5×10^{-8} M and 5×10^{-7} M) caused a dose dependent reduction of Δt by 9±2 ($p<0.05$) and 31±5 per cent ($p<0.001$) respectively ER was not affected

d-propranolol (5×10^{-7} M) had in contrast to dl-propranolol no effect on Δt. Since d-propranolol has the same membrane stabilizing action as dl-propranolol but much less β-adrenoceptor blocking potency the findings show that the reduction in transmitter release in response to dl-propranolol was due to β-blockade

Despite the increase in transmitter release during exposure to isoprenaline and the reduction in transmitter release during β-blockade the ER was unchanged This is an expected consequence of the narrow neuromuscular gap in the portal vein where transmitter concentration peaks of supramaximal amplitudes are obtained with each nerve impulse (Ljung 1976)

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D 37

Role of Blood-borne Catecholamines for Plasma Volume Restoration in Haemorrhage

By J HILLMAN and J LUNDVALL. *Department of Physiology University of Lund Sweden*

Mobilization of fluid from the extravascular space of skeletal muscle into the circulatory system is an important mechanism for plasma volume restoration in haemorrhage This absorption is to a great extent caused by decrease of capillary hydrostatic pressure (Pc) due to sympathetically induced increase of pre- to postcapillary resistance ratio (ra/rv) The present experiments on cat skeletal muscle indicate that the decrease of Pc mainly is mediated by the sympathetic vasomotor fibers in the early period of haemorrhage and

later is mainly linked to release of catecholamines from the adrenal glands. Activation of the β-adrenoceptors in the microcirculation seems essential for the fluid absorption.

In the autoperfused innervated muscle standardized haemorrhage (15 % of the blood volume) evoked an initial, rapid fluid absorption which gradually declined to attain within less than 5 min a steady rate, maintained during the period of observation (10–15 min). This steady state absorption was much reduced or abolished after adrenalectomy as well as after regional blockade of the β-adrenoceptors. In the cross-circulated muscle haemorrhage of the recipient caused a similar initial absorption as described in the autoperfused muscle. However this absorption was markedly attenuated or even ceased within 5 min despite maintained increase of regional resistance.

The cross-circulation experiments suggest that nerve mediated transcapillary fluid absorption in bleeding, although quite significant, only occurs during a limited period of time. This is in accordance with experiments using artificial sympathetic nerve stimulation and may be due to interference from local vascular control systems causing gradual decline of the α-adrenoceptor induced increase of precapillary resistance and return of ra/rv to control level. The importance of the adrenals for the maintenance of the steady state fluid gain into the circulatory system seems mainly related to activation of β-adrenoceptors. This β-adrenergic effect could be ascribed to dilatation of the precapillary sphincters leading to an increased number of patent capillaries available for transcapillary fluid exchange, and to decreased Pc. Such a β-adrenergic effect on Pc must imply an increase of ra/rv apparently due to a resistance vessel dilatation which, in relative terms, is more pronounced in the post- than in the precapillary vessels.

D 38

Sodium Permeability Properties of Myelinated Nerve Fibres from the Rat. Potential Clamp Experiments

By Tom Brismar. *Department of Clinical Neurophysiology Huddinge Sjukhus H186 Huddinge Swede*

The permeability properties of the nodal membrane in myelinated nerve fibres from albino rats were investigated with potential clamp technique. The method was similar to the one earlier developed for the analysis of frog myelinated nerve fibres (Dodge and Frankenhaeuser 1958). Single large fibres (10–12 μm) were isolated by microdissection from the sciatic nerves. This procedure was more difficult in the rat compared to the isolation of frog nerve fibres. The potential clamp analysis was carried out on fibres that at a resting potential of −70 to −80 mV (set by the d.c. balance control of the feed-back amplifier) had action potentials of about 100 mV and a threshold of 15 to 20 mV.

Records were made of the membrane current associated with different potential steps. At positive test pulses there was a transient initial inward current which depended on external [Na] and reversed direction at large positive steps. The current density was calculated from estimates of the nodal area (22.5 μm^2) and the internodal impedance (32.4 MΩ)

in 12 μm fibres. The amplitude of the Na current was affected by the potential preceding the test pulse Similar to the steady state inactivation (h_∞) curve in frog nerve (Frankenhaeuser 1960) a smooth symmetrical sigmoidal curve described the change in peak Na current at different conditioning potentials. The time course of the Na inactivation process was measured (in three fibres) by varying the duration of the conditioning pulse. It was exponential with a time constant that was maximal (5–7 ms) at potentials close to resting potential and minimal (0.5–1 0 ms) at large negative and large positive conditioning pulses. The following quantitative data (mean values) were extracted from measurements at 22-25°C in four fibres Na current reversal potential 44 mV maximum peak P_{Na} (constant field Na permeability) at large positive steps 4 6×10⁻³ cm s⁻¹ potential at which P_{Na} is 50% turned on −43 mV h_∞ at resting potential 0 68.

These results are in agreement with an earlier description of the Na current versus potential relation in rat nerve (Horáckova, Nonner and Stämpfli 1968) The Na permeability properties of myelinated nerve fibres have earlier been analysed in potential clamp experiments in frog nerve (cf Frankenhaeuser and Huxley 1964) The present data indicate that rat and frog myelinated nerve fibres have essentially similar Na permeability properties.

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D 39

Copper Induced Abnormal Chromatin Stability in Human Ejaculated Spermatozoa

By U KVIST and R ELIASSON *Reproductive Physiology Unit Department of Physiology Karolinska Institutet S 104 01 Stockholm 60 Sweden*

The nuclear chromatin condenses during spermiogenesis and achieves a high degree of stability when the spermatozoa pass through the male genital tract. The stabilizing process will normally be completed at the time of ejaculation by components in the seminal plasma. In the female genital tract the chromatin is then destabilized and at the time of fertilization there is a rapid decondensation, necessary for the subsequent cell divisions Therefore condensation and decondensation of the nuclear chromatin of the spermatozoa are processes of importance for fertility The role of various endogenous and exogenous factors on these processes are under investigation In this report the in vitro effects of copper on ejaculated spermatozoa will be presented

The chromatin of human spermatozoa is normally stabilized by zinc interacting with

groups. The degree of stability can be measured as resistance to various agents, e g. m dodecyl sulphate (SDS) Ejaculated spermatozoa are normally stabilized and do lecondense when exposed to SDS alone but do decondense when treated with both tal chelating agent e.g. EDTA (2 mM) and SDS (SDS-EDTA) (Kvist U To be pub-d).

pper (Cu^{++} 1 mM) can induce chromatin stability by oxidation of thiol groups into phide bridges (Bedford J M. et al. J Reprod. Fert. 1973 33 19) We have found that er (1.6 mM) induced a high degree of stability towards both SDS and SDS-EDTA. hermore, copper releases zinc from the spermatozoa.

ms copper will not only increase chromatin stability but also change the physiological dne dependent stability into a different and probably abnormal, non-zinc dependent lity Oxidation of thiol groups into disulfide bridges in the nuclear proteins and a taneous displacement of zinc from these thiol groups seems to be a plausible explana-to our results.

physiological destabilization by zinc release from the spermatozoa in the female geni-act seems to be possible since follicular fluid can induce zinc release in vitro (John-Ø and Eliason, R. Andrologia 1976. 8 283) In addition to copper we have found other oxidative agents and in vitro storage of the spermatozoa change the normal lity into disulfide bridges dependent stability Such chromatin may not destabilize in emale genital tract. It is known that aged spermatozoa have a reduced fertilizing ability also give rise to a high incidence of fetal abnormalities. Further studies how various itions can influence the chromatin stability in human spermatozoa are in progress.

D 40

osine Effects on Cholinergic Neurotransmission in the Rabbit Stomach In vivo

By L. Gustafsson P Hedqvist B B Fredholm and S. Ölundh. *Departments of Physiology and Pharmacology Karolinska Institut Stockholm Sweden*

re is evidence that adenosine inhibits the release of acetylcholine in the guinea pig n *in vitro* (Vizi and Knoll 1976 Sawynok and Jhamandas 1976 Gustafsson *et al.* 1977). presynaptic effect of adenosine is inhibited by methylxanthines and enhanced by s that inhibit adenosine inactivation, such as dipyridamol and dilazep The present y was undertaken to investigate whether a presynaptic effect of adenosine may be ent also *in vivo*

abbits anaesthetized with sodium pentobarbitone were used. The left and/or right us was cut and stimulated in the neck-region. Gastric motor responses were registered neans of a strain gauge transducer Blood pressure, heart rate and pulmonary insuffla- pressure were also recorded. A thin plastic indwelling catheter was placed in the iliac artery Drugs (acetylcholine, adenosine dilazep) were administered either via the iac artery or into the left ventricle.

lectric stimulation 1 min of the vagus (3–5 Hz, 5-8 V 1 msec duration) gave re

sem

producible contractile responses in the stomach and reductions of heart rate without significant alterations in blood pressure. The vagal effects were inhibited by atropine Acetylcholine infusion (3?–640 μg/min) into the coeliac artery produced clearcut contractile effects in the stomach with minimal effects on blood pressure or heart rate

Adenosine given into the coeliac artery (0.5–1 mg/min) did not produce any effects on blood pressure or heart rate while the same dose given into the heart typically reduced blood pressure from 80 to 30 mmHg. However when the adenosine uptake inhibitor dilazep (0 25–1 μmol/kg) had been given intraarterial adenosine produced pronounced systemic effects These results demonstrate the presence of a very active adenosine removal system which is inhibited by dilazep

Adenosine infusion into the coeliac artery inhibited the gastric response to vagus stimulation by 50% or more This effect of adenosine was enhanced by dilazep On the other hand adenosine enhanced the contractile response to intraarterial acetylcholine The fall in heart rate following vagus stimulation was not inhibited by adenosine.

The present results are compatible with the opinion that also *in vivo* adenosine might inhibit acetylcholine release from cholinergic nerve endings In addition adenosine might potentiate some postjunctional effects of acetylcholine by an unknown mechanism. A similar differential effect of adenosine on pre- and postjunctional components in adrenergic neurotransmission has been described earlier (Hedqvist and Fredholm 1976)

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D 41

In Vitro Studies of Antler Blood Vessels

By M WIKA *Dep anat Dent Fac Univ Oslo Norway and* L. EDVINSSON *Dep histol Univ Lund Sweden*

Antler development and function have been a matter of much debate Antlers have for some time been regarded as having thermoregulatory function Thermoregulation is coupled with the vascular system and may involve amine receptors. A study on innervation of the antler vascular bed has shown well innervated vessels below the antler and in all but a few occasions non-innervated vessels in the antler Antler arteries have a thick muscular wall with a longitudinally directed inner layer and a circularly oriented outer layer The adventitia contains mast cells which may influence circulation.

Pieces of blood vessels from domestic reindeer were studied for circular and longitudinal muscle activity in an in vitro system. The vessels were kept in ringer on ice and were found to be viable for several days.

: supplying arteries below the antler show circular muscle activity which may be
ntrated even in the set up for registration of longitudinal tensions, as an increase in
of the vessel piece. Antler arteries contract both in circular and longitudinal direc
Pieces of the vessels contract in a dose dependant way upon exposure to noradren-
adrenaline phenylephrine histamine and 5-HT The alpha adrenergic type receptor
een demonstrated for noradrenaline, adrenaline and phenylephrine by using phen-
ine, phenoxybenzamine and dibenamine as blocking agents

ces of antler blood vessels have further been found to be spontaneously and rhythmi-
active. 18 of 4 pieces tested for circular activity and 11 of 22 tested for longitudinal
e activity showed vigorous variable rhythmical contractions. In some cases this type
ivity was elicitated by pharmaca as $PGF_{2\alpha}$ or small concentrations of catechol-
s.

far we have not been able to clearly demonstrate adrenergic beta receptors in antler
l vessels.

e spontaneous rhythmic activity in antler blood vessels is in good agreement with our
g of an oscillating blood flow to the antler when measured by electromagnetic flow-
(M. Wika & J Krog in progress) In these measurements the vascular bed of the ant
part of it seem to go into spasms that reduce or completely stop the antler circulation
riable length of time. The reason for this could in certain cases be manipulations,
er cases the causes could not be identified.

D 42

nce for a Dual Effect of Intracellular Ca^{++} on Pinocytosis

By P Johansson and J -O Josefsson *Department of Pharmacology University of Lund S eden*

ion in the extracellular medium greatly influences the formation of pinocytotic chan-
in *Amoeba proteus* Micromolar concentrations of Ca^{++} stimulate while millimolar
entrations inhibit cation (Na^{+}) induced pinocytosis. The inhibitory effect reflects
petition between the inducing cation and Ca^{++} at the cell surface while the stimulating
t of Ca^{++} is excerted on some channel-forming apparatus in the cytosol (Josefsson
, 1975)

addition to these effects of Ca^{++} the following observations on pinocytosis in Ca^{++}
media indicate that passage of Ca^{++} from the cell surface into the cell or ionization
ound Ca^{++} in the cell interior may inhibit pinocytosis:

Agents which activate or increase the capacity of the cell for pinocytosis (e.g. 10^{-4}
bcAMP 10^{-4} M theophylline 2 mg/l ionophore A 23187 10^{-4} M noradrenaline, 10^{-4} M
amine) inhibit the formation of pinocytotic channels when added to the Ca^{++}-free in-
ing solution. Neither inhibition nor activation occurs in calcium-depleted cells (cells
reated with Ca^{++}-deficient media) None of the activating agents with the exception
A 23187 is inhibitory in the presence of 10^{-4} M verapamil.

2 Caffeine (0 2 mM) inhibits pinocytosis in normal cells but not in Ca^{++}-depleted cells. Procaine (10^{-4} M) or high Mg^{++} antagonizes the effect of caffeine Inhibition by caffeine is potentiated by activating agents but not affected by verapamil

3 Substitution of Cl in the inducing solution for a monocarboxylate anion (propionate, acetate lactate glycolate) decreases pinocytosis intensity The degree of blockade increases with the pK of the acid and the calcium content of the cell The mechanism of inhibition is probably release of Ca^{++} from intracellular stores Caffeine cAMP (10^{-4} M) and activating hormones potentiate blockade of pinocytosis by acetate while cGMP (10^{-4} M) insulin (10^{-4} U/l) and somatostatin (10^{-4} g/l) restore normal pinocytosis

4 Pinocytosis inhibited by Ca^{++}-depletion is normalized by activating agents caffeine or acetate but further impaired by insulin somatostatin or verapamil

In conclusion pinocytosis intensity increases when the free Ca concentration of the cytosol attains a certain level but decreases when Ca exceeds that level. This dual effect of Ca^{++} may contribute to the understanding of the transient character of cation induced pinocytosis the extremely weak inducing potency of calcium salt solutions and the complex shape of the dose-response curves of cationic inducers Mammalian hormones may either reduce (insulin somatostatin) or increase (activating hormones) the intracellular free Ca^{++} level and so modify the intensity of pinocytosis in the amoeba.

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D 43

Autonomic Control of Penile Volume in Rabbit

By N O Sjöstrand and E Klinge *Department of Physiology I Karolinska Institutet Stockholm Sweden and Department of Pharmacology University of Helsinki Helsinki Finland*

The autonomic pathways and mediators behind penile erection and relaxation have been under debate. Partly this could be due to the circumstance that no one seems to have stimulated the autonomic nerves to the penis with lower frequencies than ~0 Hz (*vide* Klinge and Sjöstrand 1974) Here some data on the relationship between penile volume and frequency of stimulation of these nerves are presented

Rabbits anesthetized with urethane were used Ureters were cannulated Bladder jejunum ileum, colon and upper part of rectum were removed Vasa deferentia were tied. The penis was skinned and placed in a greased glass plethysmograph The skin was tied around as sealing. Penile volume was recorded with a low pressure transducer on a Grass polygraph Hypogastric and pelvic nerves as well as the sympathetic chain (L6–S1) were cut and placed on stimulation electrodes Supramaximal voltage was used

Very low frequency stimulation of the sympathetic chain produced profound detumes-

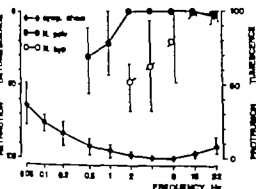


Fig. 1 Frequency-response relationship between penile volume and stimulation of the sympathetic chain and the pelvic and the hypogastric nerves. All nerve stimulations are bilateral. Each point mean and range from three expts. Frequency on logarithmic Scale.

cence of the penis (Fig. 1) The resting volume (70–90% retraction) was achieved with 0.1–0.5 Hz. Stimulation of the pelvic as well as the hypogastric nerves produced tumescence of the penis. However the two nerve sets had different frequency-response curves (Fig. 1). The response to hypogastric nerve stimulation could be affected by scopolamine (8–12 mg/kg) in rabbits seemingly devoid of atropinase while that to pelvic nerve stimulation was almost unaffected by the drug.

It is suggested that the penis is supplied with a very efficient vasoconstrictor innervation apparently operating at very low frequencies. The organ is supplied with sympathetic as well as parasympathetic dilator fibres. The former of which might—at least in part—be cholinergic while the latter might chiefly be non-cholinergic

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D 44

Cumulative Blood Flow during Reactive Hyperaemia in Human Cutaneous Tissue

By J K. KRISTENSEN and O HENRIKSEN *Depts of Dermatology and Nuclear Medicine Rigshospitalet Copenhagen Denmark*

Consider the experiment where blood flow is stopped at $t=0$, released at $t=t$ and blood flow has decreased to the preocclusion level at time t_4 then cumulative blood flow V_R, between t_1 and t_4 can be written as:

$$V = \int_{t_1}^{t_4} f(t)\,dt \quad (1)$$

where $f(t)$ is the perfusion coefficient. Applicating ^{133}Xe assuming homogeneous perfu-

sion no recirculation and washout of tracer is only flow limited then the externally monitored decrease in activity at time t, $-dq(t)/dt$ is given as

$$(-dq(t)/dt)/q(t) = f(t)/\lambda$$

where $q(t)$ is the activity at time t and λ is the tissue to blood partition coefficient. Rearranging eq 2 and integrating on both sides gives

$$-\lambda \int_{t_1}^{t_2} d\ln q(t) = \int_{t_1}^{t_2} f(t)dt = V_B \quad (3)$$

Solution of eq 3 gives

$$V_B = \lambda \ln(q(t_1)/q(t_2)) \quad (4)$$

Excess cumulative blood flow $V_{B,\,excess}$ during reactive hyperaemia can be calculated as

$$V_{B,\,excess} = \lambda \ln(q(t)/q(t)_{po}) \quad (5)$$

where $q(t_1)_{po}$ is obtained by retropolating the activity curve versus time from t_s to t_1 Relative repayment R equals excess cumulative blood flow divided by blood flow deficit calculated as the product of preocclusion blood flow and duration of vascular occlusion *i.e*

$$R = V_{B,\,excess}/(\lambda\ k_{po}\ t_1) = (\ln(q(t_1)/q(t_1)_{po}))/(k_{po}\ t_1) \quad (6)$$

where k_{po} is the washout rate constant before vascular occlusion.

Six normals were studied Cutaneous blood flow was measured dorsally on a finger by the local ^{133}Xe washout technique A cuff was placed proximally on the finger Vascular occlusion lasting from 3 to 24 min was induced by inflating the cuff to 250 mm Hg

Prolonging the duration of vascular occlusion from 3 to 12 min caused an increase in maximum cutaneous blood flow from 54 to 104 ml/100 g min Ischaemia for 24 min did not cause a further increase in maximum blood flow $V_{B,\,excess}$ (eq 5) increased from 17 ml/100 g to 100 ml/100 g following 3 and 24 min of vascular occlusion respectively Relative repayment, R (eq 6) was not correlated to duration of vascular occlusion. There was an inverse correlation between relative repayment and preocclusion blood flow

The results indicate that the hyperaemic response is at least partly due to local changes in concentrations of different metabolites liberated in the tissue The inverse correlation between relative repayment and duration of vascular occlusion suggests that besides the nutritive demand other factors such as heat regulation has influenced preocclusive blood flow in cutaneous tissue

D 45

Sleep in Cats after a New 5-HT Uptake Inhibiting Drug—Lu 10-171

By T Kovala A Leppävuori and P Putkonen *Institute of Physiology University of Helsinki Finland*

Lu 10-171 is a bicyclic phthalane derivative with potent and specific 5-TH uptake inhibiting properties. In contrast to most tricyclic antidepressants it is practically devoid of NA

uptake inhibiting effects and has low anticholinesterase and antihistaminergic activity (Hyttel 1977).

The importance of 5-HT for physiological sleep is established by experiments involving depletion and replenishment, but loading with 5-HT precursors has had variable consequences.

We have studied the EEG stages of vigilance after intraperitoneal injections of 1 and 5 mg/kg of Lu 10-171 in 9 chronically implanted cats. The 16 h mean percentages (±SD) of the sleep stages are summarized below:

Table

Stage	Control NaCl (N=9)	Lu 10-171 1 mg/kg (N=5)	Lu 10-171 5 mg/kg (N=6)
Awake (A+D)	30.6±7.9	25.2±8.0	23.6±6.0
Aroused (A)	11.6±5.6	9.5±4.3	8.5±3.0
Drowsy (D)	19.0±5 6	15 6±5 4	15 1±6.4
Slow wave sleep (S1+S2)	53.5±6 4	62.8±11 0	74 1±5.6
Light SWS (S1)	23.8±3 7	23.2±8.5	20.7±5.5
Deep SWS (S2)	29.6±5 4	39.6±8.8*	53 4±7.4**
Paradoxical sleep (PS)	15.9±4.9	12.1±3.9	2.3±1.2**

*p<0 02, ** p<0 001 Difference from control

The main results were: 1) A dose dependent increase in deep SWS 2) Inhibition of PS with the higher dose. Other stages, especially arousal decreased moderately.

The tricyclic antidepressants which inhibit both 5-HT and catecholamine uptake are known as potent suppressors of PS. Lu 10-171 differs from them in having weaker PS inhibiting effects and promoting specifically deep SWS.

Administration of 5-HT precursors has lead to contradictory results including both increase and decrease of PS in man. In the cat (Ursin 1976) 5-HTP moderately inhibited PS, but, at variance with Lu 10-171 increased the synchronized drowsy pattern instead of SWS. Our results, however are more in agreement with Autret *et al* (1976) describing decrease in PS and increase in deep SWS (stages 3+4) in humans after repeated high doses of 5-HTP.

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D 46

A Possible Role of Endocytosis in Lysosomal Activation in Dystrophic and Denervat Skeletal Muscle

By R LIBELIUS *Department of Pharmacology University of Lund Sweden*

Previous studies have shown that a cobra α neurotoxin (Naja naja siamensis Mw 782 binds specifically to cholinergic receptors and is taken up intracellularly by mouse skelet muscle *in vitro* (Libelius 1974) *In vitro* intracellular uptake of toxin (+37°C) was foun to be stimulated by cationic proteins e g. protamine and is believed to occur by endo cytosis (Libelius 1975) In addition biochemical and ultrastructural examination showe that protamine caused lysosomal activation with autophagic vacuolation of muscle fibers Protamine also stimulated intracellular uptake of another macromolecule horseradish per oxidase in mouse skeletal muscle fibers under *in vitro* conditions (Jirmanová et al 1977)

Since endocytosis and lysosomal activation in response to protamine *in vitro* occu concomitantly in skeletal muscle a causal relation between the two phenomena seeme possible Therefore it was of interest to study endocytosis in dystrophic and denervate muscles both of which display increased activities of lysosomal enzymes (Weinstock and Iodice 1969) Endocytosis *in vitro* and *in vivo* was studied by a combination of biochemi cal radiochemical and light and electron microscopic methods using ^{3}H-inulin and horse radish peroxidase as macromolecular tracers

In vitro experiments showed that the uptake of both ^{3}H inulin and horseradish per oxidase at +37°C was increased in leg (extensor digitorum longus and soleus) muscles denervated for 6 days and in leg muscles from hereditary dystrophic mice (Bar Harbor 129 strain) when compared with innervated and littermate control muscles The uptake of i v injected horseradish peroxidase measured biochemically was likewise increased in denervated and dystrophic muscles When horseradish peroxidase was injected i.v light microscopic examination of the muscles showed that the tracer was present not only in the extracellular space but also as deposits within denervated and dystrophic muscle fibers Such deposits of horseradish peroxidase which were not found in normal muscle fibers were found by ultrastructural examination to be membrane limited and were lo cated between myofibrils close to labelled transverse tubules Primary endocytic vesicles containing horseradish peroxidase appeared to originate from the sarcolemma and the transverse tubules The results suggest an increase in endocytic activity in dystrophic and denervated skeletal muscle with distribution of exogenous tracers into secondary lyso somes

Endocytosis regardless of what initiates it may constitute an important mechanism of lysosomal activation and therefore of muscle fiber degeneration

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D 47

...trol of Lipolysis and Circulation in Adipose Tissue by Endogenous Adenosine

By BERTIL B. FREDHOLM and ALF SOLLEVI. *From the Department of Pharmacology Karolinska Institutet Stockholm Sweden*

...pathetic nerves are the most important influence on adipose tissue circulation and ...bolism. In addition there are local mechanisms regulating adipose tissue function. ...nosine seems to be important in this respect. The release of adenosine from isolated ...cells in vitro (Schwabe *et al.* 1975) and from intact adipose tissue by nerve stimulation ...holm 1976) was recently reported. Adenosine is a potent inhibitor of noradrenaline ...ced cyclic AMP elevation and lipolysis in fat cells (Schwabe *et al* 1975 Fredholm ...7), and it inhibits lipolysis due to nerve stimulation *in vivo* both by a prejunctional and ...junctional effect (Hedqvist and Fredholm 1976) The possibility therefore exists ... adenosine released during nerve stimulation modulates adipose tissue function by pro... postjunctional actions. In the present communication results with two drugs that in... adenosine inactivation are presented

...nine subcutaneous adipose tissue with intact blood and nerve supply was studied. ... effect of an inhibitor of adenosine uptake dilazep (Asta Werke Brackwede BRD) ... an inhibitor of enzymatic adenosine breakdown erythro-9-(2-hydroxy 3-nonyl)-adenin ...INA, Wellcome Research Triangle Park, N C.) on glycerol release and vasoconstric... during nerve stimulation (4 Hz) was studied.

...either dilazep nor EHNA had any effect *per se* on lipolysis in isolated fat cells (Fred... 1977). Dilazep (5–20 μM) caused a 60% reduction in glycerol release induced by ...ve stimulation *in vivo* (n=6, p<0 01) without affecting basal lipolysis. Similar results ...re reported for a different adenosine uptake inhibitor dipyridamol (Fredholm and Sol... 1977). The maximal vasoconstrictor response to nerve stimulation was inhibited and ...autoregulatory escape during a 15 min stimulation was enhanced.

...HNA (5–10 μM) did not alter maximal vasoconstrictor effects but significantly poten...ed the autoregulatory escape. Lipolysis was unchanged or even slightly enhanced in ...allel with the improved circulation.

...irect measurement of adenosine deaminase showed that the fat cells had only 5% ...the total tissue activity although they contribute more than 90% of the cell mass. On ... other hand fat cells had an avid adenosine uptake system with high capacity and an ...nity in the μmolar range

These findings show that different cellular components of the same tissue may inactivate ...enosine by different mechanisms. Our results are also compatible with the opinion that ...logenous adenosine may be involved in the autoregulatory escape in adipose tissue and ...as endogenous modulator of lipolysis.

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D 48

Reflex Adrenergic Control of Pancreatic Hormone Release

By J JÄRHULT S INGEMANSSON and J HOLST *Departments of Physiology and Surgery University of Lund Sweden and Department of Clinical Chemistry Bispebjerg Hospital Copenhagen Denmark*

It has recently been described that unloading of the carotid baroreceptors is associated with a large increase in the blood glucose concentration whereas stimulation of the carotid chemoreceptors had only minor effects on the blood glucose level (Järhult, Holmberg and Lundvall 1977) It is reasonable to believe that such an hyperglycemic response evoked from arterial baroreceptors also involves an adjustment of the release of the pancreatic hormones and the present study was outlined to test this hypothesis

The effects of unloading of the carotid baroreceptors on arterial plasma glucose concentration as well as on portal plasma immuno-reactive glucagon (IRG) and insulin (IRI) concentrations were studied in anesthetized vagotomized cats by sectioning of the sinus nerves or in some experiments by lowering the pressure in the isolated carotid sinuses Cervical vagotomy *per se* slightly decreased the basal IRI concentration and increased the basal IRG concentration whereas plasma glucose remained unchanged Complete elimination of the carotid baroreceptor discharge by sectioning of the sinus nerves caused a doubling of plasma glucose and an increase in IRG by about 200% whereas IRI decreased to about 50% of its control value These baroreceptor-induced metabolic and hormonal changes which were maintained during 45 min of observation showed to be mediated mainly by the sympatho-adrenal system since cutting of the splanchnic nerves above the adrenal glands abolished the hyperglycemia and hypoinsulinemia and markedly depressed the hyperglucagonemia. Bilateral adrenalectomy only suppressed the magnitude of these reflex responses whereas sympathectomy of the pancreas virtually abolished them The pancreatic hormonal adjustments seemed to be graded in relation to the drop in carotid blood pressure and they were clearly detectable when the distending pressure in the carotid sinuses was lowered from 120 to 95 mm Hg —An interesting observation was that i v infusion of adrenaline (1 μg/kg×min) caused hyperglycemia, hyperglucagonemia and hypoinsulinemia whereas infusion of noradrenaline in the same dose raised the IRG concentration twice but did not significantly alter the IRI or glucose concentrations

We conclude that the release of insulin and glucagon can be strongly influenced by

-iations in the arterial blood pressure This reflex control of endocrine pancrease is -erted from arterial baroreceptors and mediated to the gland by the sympatho-adrenal -tem. The reflex seems particularly dependent on a direct sympathetic innervation of -e islets of Langerhans.

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D 49

-e of Muscle Mass in the Cardiovascular Response to Isometric Contractions

By Bengt Saltin J H Mitchell, B Schibye and F C. Payne. *August Krogh Institute University of Copenhagen Copenhagen Denmark*

-dynamic concentric exercise the cardiovascular response is related to work intensity -d muscle mass involved in the work. In contrast in isometric contractions the enhance-ment of blood pressure (BP) and heart rate (HR) has been shown to be solely a function of the relative exercise intensity and independent of muscle mass involved (Lind and McNicol 1967 McCloskey and Streatfield 1975) When we repeated similar studies we were unable to confirm the independency of the muscle mass for the magnitude of the BP and HR elevation while performing sustained contractions.

Eleven men were studied performing at 40% of maximal voluntary contraction (% MVC) for two minutes with digits II and III (d) handgrip (h) knee extension (k) and combining (h) and (k) Blood pressure was measured directly (a. brachialis) and recorded together with an ECG and the force output with a Brush recorder Already early during the contraction the lowest elevation in HR and BP was observed for (d) and the largest for (h) and (k). During the latter phase of the contractions the following values were observed (mean and SD): HR 83.8±12.9 (d) 102.4±14 0 (h) 117 4±19.2 (k) 123.5±18.7 (h+k) BP 153.4±18.7 (syst.) 103.3±12.5 (diast.) (d) 186 4±19 7 128.5±13.8 (h) 206.6±19.6, 142.1±12.5 (k) 212.0±29 6, 153 4± 1.5 (h+k) The differences in HR and mean BP comparing the various muscle synergies were significant.

The rather large individual variation in HR and BP responses was due to a certain day to day variation and to the finding of a greater elevation of HR and BP the larger the absolute force developed in the contraction. In fact combining all individual data a close relationship was found for the developed absolute force and the HR and BP responses (r=0.9 p<0.001). The only explanation we can give for the discrepancy between our and earlier results is that in previous studies the contractions have not been confined to isolated muscle groups and the variation in muscle mass has not been as large as anticipated. To what extent central and peripheral factors contribute in the regulation of HR and BP cannot be stated. By applying cuffs and inflate them to above arterial pressure at the point of termination of the contraction, BP stayed elevated, suggesting a peripheral chemosensitive receptor playing a role in the regulation.

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D 50

Utilization of Dietary Amino Acids by Grouse (Lagopus Lagopus)

By A. Mortensen and A Tindall. *Department of Physiology University of Tromsø Norway*

It has been suggested that the function of the caeca in birds is cellulose digestion (McBee and West, 1969) An alternative idea is that they are important in nitrogen metabolism by the catabolism of some urinary nitrogenous compounds and reutilization of the nitrogen moiety Barnes (1972) found organisms capable of uric acid metabolism in the caeca of fowl but none capable of digesting cellulose

We have considered the problem from several angles 1 We have examined the amino acid content of both artificial and natural foods and of the contents of the ileum and caeca, and also the faeces 2. We have looked at the absorption of labelled amino acids injected into the caeca and 3 we have done some work on the breakdown of uric acid in the caeca.

Under equithesin anaesthesia, the abdomen was opened and by means of a soft catheter 0 1 mg amino acid mixture (activity=50 μCi/ml) in a volume of 1 ml was injected being spread throughout a length of several centimetres of one caecum Blood samples from the wing vein taken at subsequent intervals showed marked rises in radio-activity which indicated absorption from the caecum. It cannot be concluded that such activity detected in the blood is necessarily the labelled amino acids since they may have been catabolized while in the caecum However the very short time lag between injection and appearance of activity in the peripheral circulation makes this unlikely

When uric acid is broken down the carbon atoms are normally removed and excreted as CO_2 while the nitrogen-containing portion may be reused after being degraded further even to ammonia. Concentrations of ammonia in the caeca and colon were found to be substantial and in precursory experiments we have incubated 0 5 g caecal contents with 0 02 ml of a solution of ^{14}C-labelled uric acid (activity=50 μCi/ml) The released CO_2 was radio-active

Thus the experiments suggest that either amino acids or their immediate breakdown products are readily absorbed from the caeca We also think that uric acid is catabolized so that the nitrogen-containing part can be reutilized but further work is required to establish this idea or not.

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D 51

Release of Adenosine-like Material from Isolated "Perifused" Fat Cells

By P. Hjemdahl, B. B. Fredholm and A. Sollevi, *Department of Pharmacology Karolinska Institutet, Stockholm, Sweden*

Adenosine, a breakdown product of adenine nucleotides has been ascribed a role as endogenous modulator of cyclic AMP accumulation and lipolysis in isolated fat cells (cf. Schwabe *et al.* 1975). Adenosine accumulates in fat cell incubates (see Schwabe *et al* 1975) and is released from adipose tissue *in vivo* (Fredholm 1976). The kinetics of adenosine formation and breakdown are however incompletely known.

^{3}H-adenine or ^{3}H-adenosine was taken up by isolated fat cells and incorporated into nucleotides. The uptake and phosphorylation followed Michaelis-Menten kinetics with a V_{max} of about 2 nmoles × min^{-1} × g^{-1} and a K_m in the μmolar range. After labelling the cells were placed in a temperature-controlled plastic chamber and perifused according to Allen *et al.* (1973) with 2 ml buffer/min. This technique allows continuous removal of products formed by the fat cells. The basal rate of release of radioactivity was 0.49±0.04% of the cellular content per min (n=11). Most of the radioactivity released was nucleosides of which adenosine was a major component. Assuming homogenous labelling of the nucleotide stores the rate of adenosine release is calculated to be 0.3–0.4 nmoles × min × g^{-1}. The release rate was decreased by 31±8% ($p<0.05$) by perfusing with oxygenated rather than aerated buffer. On the other hand, lipolytic stimuli did not significantly alter (a 12±6% increase) the nucleoside outflow. These findings are in agreement with results obtained *in vivo* and suggest that adenosine release is regulated by oxygen supply and demand but not by lipolysis *per se*.

In contrast to the situation in incubated fat cells adenosine deaminase failed to increase lipolysis in the perifused fat cell system. Exogenous adenosine was more antilipolytic in perifused than in incubated fat cells. These differences may be explained by an accumulation of adenosine in conventional fat cell incubates.

The present results support the contention (Fredholm 1976) that adenosine accumulation in adipose tissue *in vivo* may be caused by blood flow restriction and hypoxia rather than by lipolytic stimulation. The results are also compatible with the opinion that adenosine is an endogenous modulator of lipolysis *in vitro* and *in vivo*.

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D 52

The Effect of Progesterone on the Spontaneous Interictal Spike Evoked by Topical Application of Penicillin to the Cat's Cerebral Cortex

By S. Landgren, T. Bäckström and G. Kalistratov, *Department of Physiology University of [illegible], Sweden*

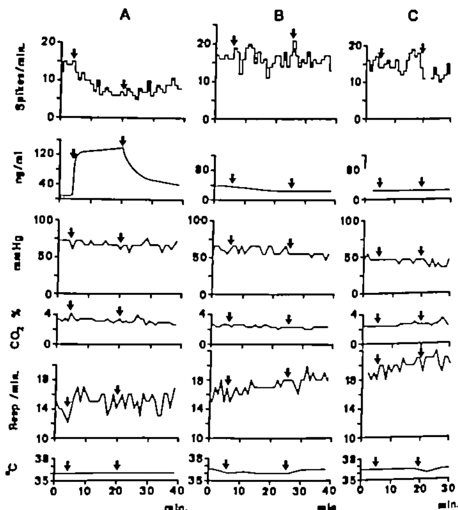


Fig. 1 Columns of graphs (A–C) showing from above downwards: the frequency of interictal penicillin spikes the plasma concentration of progesterone the mean systemic blood pressure PCO_2 respiratory rate and rectal temperature. A. Intravenous infusion of progesterone B control infusion of normal cat serum C Infusion of Mebumal 0 11 mg/ml. The graphs were obtained from three different foci A, B and C in the same animal. The chronological order was A B C. The arrows mark the beginning of the priming dose and the end of the infusion

The frequency of seizures in female epileptic patients is positively correlated to the quotient of estrogen/progesterone in their plasma (Bäckström 1976) Previous investigations indicate that estrogen may have epileptogenic effects The role of progesterone is, however less well understood. We have therefore studied the effect of intravenous progesterone infusions of low concentrations on the discharge of a penicillin focus in the cerebral cortex of the ovariectomized cat.

Filter paper (4 mm²) soaked in Na-benzylpenicillin 100 000 IU/ml were placed on the cortical surface near the post cruciate dimple A focus discharging spontaneous interictal spikes at a quasi-regular frequency was established within 10 minutes The spikes were recorded from the cortical surface near the focus Recordings of electrocorticogram ar

...ial blood pressure, end tidal PCO_2, respiratory rate and rectal temperature was made on Siemens Elema Mingograf. Progesterone dissolved in serum from ovariectomized cat or ...lonal (ACO) diluted with physiological saline were injected intravenously. A priming ...ose (1.5 ml) was followed by a slow constant infusion (11 ml/h). Blood samples were taken ...efore, during and after the infusion and the plasma progesterone was assayed using a ...irect radio-immunoassay method described by Carstensen and Bäckström (1976).

The effect of progesterone infusion is shown in Fig. 1. In 10 out of 11 experiments a ...ecrease in the frequency of the spontaneous interictal discharge was observed. Considerable effects were seen with plasma concentrations of progesterone between 40 and 70 ng/ml. Infusion of Mebumal in equimolar doses was less effective. The amplitude of the negative penicillin spike was reduced and the spike disappeared more rapidly during progesterone infusion than in control experiments.

It was concluded that progesterone in physiological plasma concentrations influenced the generation of the interictal penicillin discharge.

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D 53

Diurnal Rhythm of Hypothalamic TRF in the Rat

By J. Leppäluoto, F. Koivusalo and R. Kraama, *Department of Physiology, University of Oulu, Finland*

We have previously shown that the highest serum TSH levels occur in the middle of the light period in rats (Leppäluoto, Ranta and Tuomisto 1974). To determine if these high TSH levels might be correlated with hypothalamic TRF secretion we have measured TRF contents of rat hypothalami at various times of the light–dark period.

Rats were adapted to a silent room for 2 weeks, lights on from 6 AM to 6 PM. Six animals were decapitated at 4 hrs intervals and hypothalami and various brain parts were rapidly excised, weighed, extracted to methanol and purified in cation exchange chromatography. The eluates were assayed in TRF radioimmunoassay. In male rats higher hypothalamic TRF contents were found during the light than dark period (380±65 vs. 160±66 pg/mg of tissue, $p<0.01$). Pineal TRF contents followed the same rhythm (13 vs. 5 pg/mg) but pituitary and pancreatic TRF did not show any significant rhythm. In female rats we did not find any changes in hypothalamic TRF concentration during the light and dark period.

Although there is no correlation between the hypothalamic content of TRF and plasma TSH level in cold-exposed or thyroid-treated rats (Bassiri and Utiger 1972), there seems to be a positive correlation between those variables in relation to the time of day in male

rats according to our present and earlier results. At the moment it is not possible to if pineal and hypothalamic TRF and plasma TSH in male rats are in causal relationship

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D 54

Diurnal Variation of Hypothalamic LRF Concentration in Female Rats

By J LEPPÄLUOTO M TUOMINEN and H SELÄNNE, *Department of Physiology University of Oulu Finland*

Plasma level of LH in the female rat is a function of the time of estrous cycle and day that highest levels are obtained in the afternoon of proestrus. This would indicate that hypothalamic LRF is secreted before or in the afternoon of proestrus. We have therefore measured hypothalamic LRF concentrations in various times of cycle and day

Female rats were housed for 2 weeks in a silent room with lights on from 6 AM to 6 PM. Six rats were rapidly decapitated at 4 hrs intervals Hypothalami around the median eminence and between mamillary bodies and optic chiasma were rapidly excised, extracted with methanol evaporated dissolved in buffer and measured in a specific radioimmunoassay for LRF (Seppälä Ranta and Leppäluoto 1974) Vaginal smears were also taken and studied under light microscope

Highest median eminence LRF concentrations were found at 4 PM, 794±141 pg LRF per mg of wet tissue in diestrus and lowest at midnight 308 pg/mg ($p<0.05$ to 4 PM values) and at 4 AM 238 pg/mg ($p<0.02$) in estrus or in metestrus. Hypothalamic LRF concentrations were significantly lower and varied from 25 to 40 pg/mg without showing any significant diurnal fluctuation.

Low median eminence LRF concentration during the dark period of the day might indicate decreased synthesis of LRF in hypothalamus or increased secretion of LRF from median eminence in the dark Unaltered LRF concentrations in the rest of the hypothalamus might favour the latter possibility

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SEPPÄLÄ M. T RANTA and J LEPPÄLUOTO In: *Biological and clinical aspects of reproduction. Excerpta Medica International Congress Series No 394* Excerpta Medica, Amsterdam 1974 465–471

D 55

Effect of Sympathectomy on Active Transport Mechanisms in Choroid Plexus *In Vitro*

By B. Wrolade, L. Edvinsson and M. Lindvall. *From the Department of Pediatrics St. Görans Hospital Karolinska Institute Stockholm and the Department of Histology University of Lund Lund, Sweden*

The choroid plexuses (CP) have at least two main functions production of cerebrospinal fluid (CSF) and active transport of ions and polar compounds between blood and CSF The CP are richly supplied with both adrenergic and cholinergic nerve fibres (Edvinsson et al., 1975). Recently sympathectomy was shown to increase CP carbonic anhydrase activity and to enhance CSF production (Edvinsson, *et al.* 1975 Lindvall, Edvinsson and Owman, 1977) whereas sympathetic nerve stimulation reduced the CSF formation (Lindvall, Edvinsson and Owman, 1977). In order to further analyse the physiological role of the sympathetic nerves to the CP the effect of sympathetic denervation was studied on active transport mechanisms in isolated CP

The uptake of choline (organic base) and of PAH (organic acid) into CP of the lateral ventricle of rabbits was studied *in vitro* at one and two weeks after bilateral and unilateral superior cervical sympathectomy (SE) The CP from the non-operated side from unilaterally denervated animals and CP from non-operated animals served as controls.

The CP tissue-medium ratios (T/M) for choline was increased on the operated side by between 1.25 to 3 75 times one week after unilateral SE. One week after bilateral SE T/M for choline on both sides were of the same magnitude as those of the operated side one week after unilateral SE. SE did not change the uptake of PAH. Furthermore no side to side difference in PAH or choline T/M values was noted one week after preganglionic SE or two weeks after postganglionic SE. Total and Na K -activated ATPase activity was not significantly different between the operated and the non-operated side one week after SE.

In conclusion, SE temporarily increases CP uptake of the organic base choline *in vitro* whereas the uptake of PAH and the ATPase activity remained unchanged. Ventricular perfusion experiments may reveal if this reflects a sympathetic regulation of CSF transport mechanisms.

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rats according to our present and earlier results At the moment it is not possible to say if pineal and hypothalamic TRF and plasma TSH in male rats are in causal relationship

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D 54

Diurnal Variation of Hypothalamic LRF Concentration in Female Rats

By J LEPPÄLUOTO M TUOMINEN and H SELÄNNE *Department of Physiology, University of Oulu Finland*

Plasma level of LH in the female rat is a function of the time of estrous cycle and day so that highest levels are obtained in the afternoon of proestrus This would indicate that hypothalamic LRF is secreted before or in the afternoon of proestrus. We have therefore measured hypothalamic LRF concentrations in various times of cycle and day

Female rats were housed for 2 weeks in a silent room with lights on from 6 AM to 6 PM. Six rats were rapidly decapitated at 4 hrs intervals Hypothalami around the median eminence and between mamillary bodies and optic chiasma were rapidly excised extracted with methanol evaporated dissolved in buffer and measured in a specific radioimmunoassay for LRF (Seppälä Ranta and Leppäluoto 1974) Vaginal smears were also taken and studied under light microscope

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D 56

The Time Course of Cyclic Nucleotide Changes in Anaphylactic Guinea Pig Lung

By A. A. MATHÉ and R. J SOHN *Departments of Psychiatry Boston University and Physiology Karolinska Institutet Stockholm Sweden*

The chemical mediators of anaphylaxis affect the level of cyclic nucleotides and, conversely nucleotides influence the release of mediators Since it has been hypothesized that a fall in cAMP is necessary for the mediators release and that cAMP and cGMP change in opposite directions the time course of these two nucleotides and the release of histamine and PGs immediately following anaphylactic challenge was studied

Guinea pigs were prepared as previously described (Mathé *et al* 1975 1977) Following a rinse period two schedules were used 1 the lungs were perfused with Tyrode s and 50 mg ovalbumin injected from 0 to 10 sec 2 aliquots of minced lungs were placed in nylon mesh bags pre-incubated and then transferred to tubes containing buffer with/ without 100 μg/ml ovalbumin In both schedules after the appropriate time period, lung samples were frozen in liquid N_2. Histamine was determined fluorometrically and PGs and nucleotides by radioimmunoassays

In perfused lung, anaphylaxis caused an initial cAMP peak, followed by a decrease and a new rise until the end (Table 1) Pattern of cGMP changes was similar to that of cAMP The nucleotide levels in sensitized lungs did not change with time

TABLE 1 The time course of changes in cyclic nucleotides in perfused guinea pig lungs during anaphylaxis

Time (sec)	0	10	20	30	40	70	130	250
cAMP	2 1	2.2	5.6**	3 1	1 0*	4.5	7.2	7.9*
cGMP	0.17	0.18	0.20	0 29*	0.10*	0 49**	0.50*	0 73

Results expressed as mean nmol nucleotide/g wet wt. N=5 and denote P<0 05 and <0.01 respectively compared to time 0

Histamine PGF2α and 15-keto-13 14-dihydro-metabolite release into the perfusate at 250 sec was 25 1% 33.3 ng and 167 2 ng respectively (P<0 01 for all mediators)

In minced lung, cAMP peaked at 5 to 10 sec (150 and 136% respectively of time 0, P<0 01) did nt differ from baseline at 15 30 and 45 and was several-fold increased at 90 120 and 240 sec Changes in cGMP followed a similar pattern. Histamine and PGF2α in the media were elevated from 15 sec (436 and 285% respectively of baseline P<0 01) and until the end

The data show that the onset of anaphylaxis results in a rapid and transient increase in cAMP and cGMP The patterns of cAMP and cGMP changes are parallel The results further confirm our finding that both nucleotides are elevated in later stages of anaphylaxis" (Mathé *et al* 1974 1977) but do not support the hypotheses that a fall in cAMP is necessary for the release of mediators and that cAMP and cGMP change in opposite directions.

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The following symbols and units, recommended by the SI are being used in Acta Physiologica Scandin Certain units, not included in SI, will still be permitted.

SI units with recommended symbols

Units	*Symbols*
kilogramme	kg
second, millisecond	s ms
mole, millimole, micromole, nanomole, picomole	mol mmol μmol nmol pmol
meter millimeter micrometer nanometer	m mm μm nm
candela	cd
steradian	sr
hertz (frequency)	Hz (s^{-1})
newton (force)	N (kg m/s^2)
pascal (pressure)	Pa (N/m^2)
joule (energy)	J (N m)
watt (effect)	W (J/s)
lumen (lightflow)	lm (cd sr)
lux (illumination)	lx (lm/m^2)

Conversion factors to be given in Methods.

Permitted non-SI units

Units	*Symbols*
gramme	g
minute	min
hour	h
molarity (mol/liter)	M
(calorie)	cal (4.184 J)
(kilopond)	kp (9.81 N)
(millimeters of mercury)	mm Hg (1.333 bar)
(millibar)	mbar (100 Pa)
curie	Ci
liter milliliter microliter	l ml μl
degree Celsius	°C